Mating behavior as non-invasive biomarker in *Xenopus laevis* for the assessment of endocrine disrupting compounds

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Zusammenfassung

Viele Schadstoffe, die in die Umwelt gelangen, können mit dem endokrinen System von Vertebraten interagieren und deren Physiologie und Entwicklung nachhaltig schädigen. Weil solche endokrinen Disruptoren (EDs) in Oberflächengewässern akkumulieren, sind es vorrangig aquatische Vertebraten, wie Fische und Amphibien, die Stoffen, welche als exogene Steroide agieren oder endogene Reproduktionshormone beeinträchtigen, ausgesetzt sind. Nichtsdestotrotz benutzt man erst seit Kurzem Frösche, wie den Südafrikanischen Krallenfrosch *Xenopus laevis,* als Modellorganismus zur Beurteilung von EDs, vor allem von EDs mit (anti)androgenen und (anti)östrogenen Wirkungsweisen. Bei den existierenden Biomarkern in *X. laevis* handelt es sich jedoch stets um invasive Techniken. Nicht-invasive Methoden zur Beurteilung der EDs, wie z.B. eine kurzzeitige Exposition adulter Tiere, die zu reversiblen Effekten führt, gibt es bisher nicht.

Vorangegangene Studien zeigen, dass das Paarungsrufverhalten von männlichen *X. laevis* von Androgenen und Gonadotropinen abhängig ist. Dementsprechend könnte dieses Verhalten als Grundlage für die Entwicklung eines nicht-invasiven Biomarkers zur Detektion von EDs, speziell EDs mit (anti)androgenen und (anti)östrogenen Wirkungsweisen, welche die Reproduktion beeinflussen, dienen. Folglich ist das Ziel dieser Studie herauszufinden, ob umweltrelevante Konzentrationen (anti)androgener und (anti)östrogener EDs das Paarungsrufverhalten von männlichen *X. laevis* beeinflussen und ob dieses Verhalten als nicht-invasiver Biomarker für die Beurteilung solcher EDs herangezogen werden kann. Aus diesem Grund wurden männliche *X. laevis* gegenüber (anti)androgenen und (anti)östrogenen EDs in diversen Konzentrationen (10^{-6} M, 10^{-8} M, 10^{-10} M) exponiert. Als Modellsubstanzen wurden das androgene 17α-Methyldihydrotestosteron (MDHT), das antiandrogene Fungizid Vinclozolin (VIN), das östrogene 17α-Ethinylestradiol (EE2) und das antiöstrogene Tamoxifen (TAM) benutzt. EE2 wurde zusätzlich in einem weiteren Testlauf in den Konzentrationen 10^{-10} M, 10^{-11} M and 10^{-12} M getestet. TAM wurde einzeln in den oben genannten Konzentrationen und zusätzlich in einer simultanen Behandlung mit EE2 getestet. Ebenso wurde der reine Östrogen-Antagonist Fulvestrant (ICI) in einem simultanen Expositionsexperiment mit EE2 untersucht. Um

die verschiedenen Wirkungsweisen der EDs (androgen, antiandrogen, östrogen und antiöstrogen) identifizieren und unterscheiden zu können, sowie um den Grad der sexuellen Erregung der Tiere festzustellen, wurde eine detaillierte Analyse der unterschiedlichen Ruftypen entwickelt. Des Weiteren wurden Tests, die die Reversibilität potentieller ED-Effekte, sowie die Attraktivität der veränderten Rufe gegenüber weiblichen Artgenossen untersuchen, durchgeführt, um so die biologische Relevanz der Experimente zu bewerten.

Die Behandlung mit androgenem MDHT in allen getesteten Konzentrationen führte zu einer erhöhten sexuellen Erregung der Tiere, angezeigt durch höhere Anteile an geäußerten Werberufen und niedrigeren Anteilen des Ruftyps Rasping, welcher einen sexuell nicht-erregten Zustand des Männchens kennzeichnet. VIN und EE2 exponierte Frösche (in allen getesteten Konzentrationen) zeigten dahingegen eine erniedrigte sexuelle Erregung: Tiere der beiden Behandlungsgruppen vokalisierten geringere Prozentsätze an Werberufen. Expositionsbehandlungen gegenüber allen getesteten Konzentrationen an VIN erhöhten noch dazu den Anteil des Ruftyps Growling, während EE2 den Anteil an Rasping-Rufen steigerte. Zudem vokalisierten Männchen, welche VIN (10^{-6} M) ausgesetzt waren, generell weniger als Kontrolltiere. Eine reine TAM Exposition beeinflusste keinen der gemessenen Parameter. Waren die Tiere allerdings simultan EE2 und TAM bzw. EE2 und ICI ausgesetzt, neutralisierten die Antiöstrogene die östrogen-induzierten Effekte.

Eine Behandlung mit EE2 oder VIN, nicht jedoch die Exposition gegenüber MDHT oder TAM, resultierte außerdem in veränderten zeitlichen und spektralen Parametern des Werbegesanges von *X. laevis*. Eine simultane Behandlung mit EE2 und ICI neutralisierte die östrogenen Effekte vollständig, während eine Co-Exposition gegenüber EE2 und TAM nur einige der östrogen-induzierten Parameter aufhob. Weibchenwahlversuche ergaben, dass durch EE2 Exposition veränderte Werberufe für weibliche *X. laevis* deutlich unattraktiver sind als Rufe von Kontrollfröschen. Diese zeitlichen und spektralen Veränderungen der Werberufe verschwanden jedoch nach 6-wöchiger Haltung der Männchen unter Kontrollbedingungen. Im Gegensatz zu den verhaltensphysiologischen Effekten, zeigte nur einer der klassischen, molekularbiologischen und biochemischen Biomarker einen Effekt: EE2 induzierte die mRNA Expression von hepatischem Vitellogenin, jedoch nur in den

Konzentrationen 10^{-6} M und 10^{-8} M EE2. Geringere EE2 Konzentrationen zeigten keinen Effekt mehr.

Zusammengefasst kann die hier vorgestellte verhaltensphysiologische und damit nicht-invasive Methode durchaus als höchst sensitiver Biomarker für die Detektion von (anti)androgenen und (anti)östrogenen EDs verwendet werden und sogar zwischen den verschiedenen Wirkungsweisen differenzieren. Darüber hinaus ist die hier etablierte Methode schnell (1 – 4 Tage) und vergleichsweise kostengünstig. Ferner zeigt die hohe Sensitivität des Tests, sowie die Möglichkeit der vollautomatischen Analyse enormer Datenmengen, dass dieser schnelle Verhaltenstest ein großes Potential hat, ein sensitiver, standardisierter und nicht-invasiver Biomarker zu werden.

Summary

Many chemical pollutants that are released into the environment can interfere with the vertebrate endocrine system. Those endocrine disrupting chemicals (EDCs) have been shown to adversely affect vertebrate development and physiology. Because EDCs accumulate in surface waters, aquatic vertebrates are main targets of a vast number of compounds acting as exogenous steroids or affecting endogenous reproductive hormones. Nevertheless, only recently the anuran *Xenopus laevis* became a model organism for the assessment of EDC effects triggered by the four principle modes of action (MOAs), (anti)androgenic and (anti)estrogenic ones. However, to date existing biomarkers for the assessment of EDCs using *X. laevis* as model species are invasive techniques. Non-invasive methods, e.g. short-term exposure of adult frogs leading to reversible effects, do not exist yet.

The male mate calling behavior of *X. laevis* was shown to depend on sex steroids and gonadotropins, thus this behavior might be used as endpoint for the assessment of EDCs, especially of (anti)estrogenic and (anti)androgenic EDCs, which affect reproductive biology. Accordingly, the aim of the study was to determine whether environmentally relevant concentrations of (anti)androgenic and (anti)estrogenic EDCs affect the androgen-dependent male mate calling behavior of *X. laevis* and whether this endpoint might be used as biomarker for the assessment of such EDCs. To address this issue, male *X. laevis* were exposed to various concentrations (10^{-6} M, 10^{-8}M and 10^{-10} M) of the androgenic 17α-methyldihydrotestosterone (MDHT), the antiandrogenic fungicide vinclozolin (VIN), the estrogenic EDC 17α-ethinylestradiol (EE2), and the antiestrogenic tamoxifen (TAM). EE2 was additionally tested in a second test series, determining its effects at the concentrations 10^{-10} M, 10^{-11} M and 10^{-12} M. TAM was tested individually at the above mentioned concentrations, as well as in a simultaneous treatment with EE2. In addition, the pure estrogen antagonist fulvestrant (ICI) was also tested in a simultaneous treatment with EE2. A detailed analysis of call types was developed that allows for identifying specific MOAs of EDCs (androgenic, antiandrogenic, estrogenic and antiestrogenic) as well as to determine levels of sexual arousal of the exposed males. Additional tests were performed concerning the reversibility of potential effects of certain EDCs on male mate calling behavior, as well as the assessment whether modifications of male

mating calls affect the attractiveness of these calls towards females. These additional experiments should reveal the biological relevance of exposure of *X. laevis* to particular, environmentally relevant EDCs.

Androgen (MDHT) treatment at all concentrations tested resulted in enhanced sexual arousal of exposed males, indicated by higher proportions of advertisement calls (AC) and lower percentages of the call type rasping, which characterizes a sexually unaroused state of the male. VIN and EE2 exposed frogs (all concentrations tested), on the other hand, were less sexually aroused than control frogs: animals of both treatments uttered lower percentages of advertisement calls. VIN treatment at all concentrations also increased the percentages of the call type growling, while EE2 increased portions of rasping. Moreover, VIN at 10^{-6} M reduced males' calling activity. Individual TAM exposure did not affect any call type, however, when males were simultaneously exposed to EE2 + TAM as well as to EE2 + ICI, EE2 effects were cancelled out. EE2 and VIN exposure further altered temporal and spectral parameters of the male advertisement call of *X. laevis*, whereas MDHT and TAM exposure did not affect these parameters. Co-exposure to ICI again cancelled out these effects, while TAM co-exposure only reversed some of these parameters. In the case of EE2, effects on temporal and spectral parameters resulted in a lower sexual attractiveness of EE2 exposed males towards females as demonstrated by female choice experiments. These effects vanished gradually within 6 weeks under control conditions. In contrast, the only classical, biomolecular and biochemical biomarker that was affected by any treatment was hepatic vitellogenin (Vtg) mRNA expression. Vtg was induced after EE2 exposure at concentrations equal or higher than 10^{-8} M. Lower EE2 concentrations, however, did not affect Vtg.

In conclusion, the present study introduced a behavioral and thus non-invasive method that can be used as a highly sensitive biomarker for the detection of (anti)androgenic and (anti)estrogenic EDCs, being the first biomarker in vertebrates that differentiates between these different MOAs. Additionally, the herewith established method is fast (1 - 4 days) and comparatively economical. Moreover, the high sensitivity and the potential to analyze vast datasets rapidly in a completely automated fashion indicate the huge potential for this rapid behavior test to become a sensitive, standardized, non-invasive biomarker with even diagnostic value.

Schlagwörter: Rufverhalten, Südafrikanischer Krallenfrosch, *Xenopus laevis*, endokrine Disruptoren, Biomarker, Östrogene, Antiöstrogene, Androgene, Antiandrogene.

Keywords: Calling behavior, South African clawed frog, *Xenopus laevis*, endocrine disrupting compounds, biomarkers, estrogens, antiestrogens, androgens, antiandrogens.

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Ω	ohm
AC	advertisement call
ad	arytenoids disc
AMV-RT	avian myeloblastosis virus reverse transcriptase
ANOVA	analysis of variance
APOA	anterior preoptic area
AR	androgen receptor
BLAST	basic local alignment search tool
°C	degree Celsius
cDNA	complementary desoxyribonucleic acid
CNS	central nervous system

C_T	cycle threshold
CTRL	control
DEPC	diethyl pyrocarbonate
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxynucleotide phosphate
DTAM	dorsal tegmental area of the medulla
E2	17β-estradiol
EDs	endokrine Disruptoren
ec	elastic cartilage
EDC	endocrine disrupting compound
EDTA	ethylenediaminetetra-acetate
EE2	17α-ethinylestradiol
EF	elongation factor-1α
EIA	enzyme immunoassay
ER	estrogen receptor
EU	European Union
EtOH	ethanol
FDR	false discovery rate
FLU	flutamide
FSH	follicle-stimulating hormone
FU	fluorescence units
g	gram
g	mean gravitational acceleration
GLMM	general linear mixed model
GnRH	gonadotropin-releasing hormone
h	hour
hc	hyaline cartilage
hCG	human chorionic gonadotropin
HPG axis	hypothalamus-pituitary-gonad axis
HVC	high vocal center

Hz	Hertz (1/s)
ICD	interclick duration
ICI	fulvestrant, also known as faslodex or ICI182780,
IQR	interquartile ranges
IU	international units
L	liter
LaGeSo	Landesamt für Gesundheit und Soziales
LH	luteinizing hormone
LHRH	luteinizing-hormone-releasing hormone
LOEC	lowest observed effect concentration
LTOR	laminar nucleus of the torus semicircularis
m	meter
M	molar
M1	2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid
M2	3',5'-dichloro-2-hydroxy-2-methylbut-3-enan-ilide
MDHT	methyldihydrotestosterone
min	minute
MIS	Müllerian inhibiting substance
MOA	mode of action
Mol	mole
mRNA	messenger ribonucleic acid
MS222	tricaine methanesulfonate
MT	methyltestosterone
N. IX-X	nerve nucleus IX-X
NOEC	no observed effect concentration
nt	nucleotides
P4	progesterone
p.a.	per analysis
PCR	polymerase chain reaction
pH	measure of acidity/basicity of an aqueous solution
poly(dT)	poly desoxythymidine
Ri	reticular formation

RIN	ribonucleic acid integrity number
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
rRNA	ribosomal RNA
rRpd	dorsal nucleus raphe
RT	reverse transcriptase
RT-PCR	reverse transcriptase-PCR
s	second
S.E.M.	standard error of the mean
STW	sewage treatment works
T	testosterone
TAM	tamoxifen
tRNA	transfer ribonucleic acid
UK	United Kingdom
US	United States
UV	ultraviolet
VIN	vinclozolin
VST	ventral striatum
Vtg	vitellogenin

1 Introduction

1.1 Endocrine disrupting compounds

Endocrine disrupting compounds (EDCs) are defined as exogenous chemicals or chemical mixtures 'that alter the structure or function(s) of the endocrine system and cause adverse effects at the level of the organism, its progeny, the populations, or subpopulations of organisms' (U.S. EPA 1998). Those ubiquitous EDCs include natural substances like phytohormones; but most EDCs are thought to be of anthropogenic origin, such as pharmaceuticals, pesticides and fungicides. EDCs can modulate the endocrine system by mimicking hormone action or blocking hormone receptors (Sonnenschein and Soto 1998; Crews et al. 2000; Tilghman et al. 2010). They can also interfere with hormone biosynthesis, metabolism and excretion and affect hormone release into the blood and blood plasma transport (Crisp et al. 1998; Crews et al. 2000; Vos 2000; Lintelmann et al. 2003). By interfering with the endocrine system of vertebrates, EDCs can adversely affect vertebrate physiology and development (Vos et al. 2000; Scott and Sloman 2004; Zala and Penn 2004; Kloas et al. 2009). Besides affecting the thyroid system (Kloas et al. 2009; Lorenz et al. 2011a), the stress hormone system (Pottinger 2003), and the immune system (Chalubinski and Kowalski 2006; Inadera 2006), EDCs can especially interfere with the hypothalamic-pituitary-gonad (HPG) axis and affect various aspects of reproduction via (anti)estrogenic and (anti)androgenic modes of action (MOA) (Scott and Sloman 2004; Zala and Penn 2004; Kloas et al. 2009; Lorenz et al. 2011b).

(Anti)androgenic and (anti)estrogenic EDCs have been the main focus of research efforts so far: short- and long-term effects of such EDCs were demonstrated in birds and mammals (Crisp et al. 1998; Crews et al. 2000; Zala and Penn 2004). However, surface waters are the main sink of EDCs, especially of mass-produced industrial and pharmaceutical chemicals. Surface waters are contaminated by surface runoff, inland drainage and sewage discharge (Falconer et al. 2006; Benotti et al. 2009). Thus, aquatic vertebrates, like amphibians and fish, are main targets of a vast number of exogenous steroids or steroid-like chemicals (Hutchinson et al. 2000; Petrovic et al. 2001) and reports about (anti)androgenic and (anti)estrogenic EDCs affecting development and physiology of aquatic vertebrates accumulate (Scott and Sloman 2004; Kloas et al. 2009; Saaristo et al. 2009, 2010a and b; Lorenz et al. 2011b).

1.2 Endocrine regulation of reproductive biology in aquatic vertebrates

In aquatic vertebrates the endocrine system is organized like in most vertebrates (Matsumoto and Ishii 1992; Kloas et al. 2009). Regarding reproductive biology, the HPG axis is the most important regulating structure (Fig. 1; Norris 2006; Sower et al. 2009).

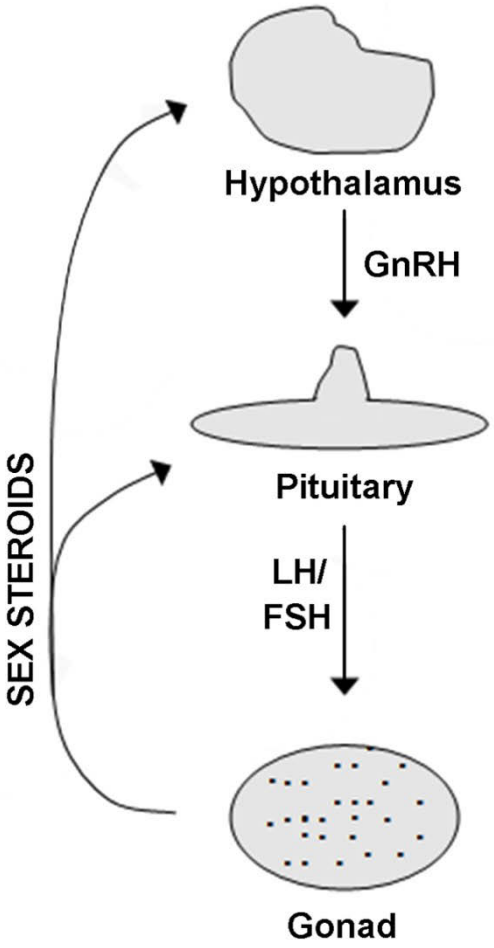

Fig. 1: The function of hypothalamus-pituitary-gonad (HPG) axis. Gonadotropin releasing hormone (GnRH) is discharged from hypothalamic central nervous system to stimulate the secretion of gonadotropins from pituitary gland. In response to gonadotropins (follicle stimulating hormone (FSH), luteinizing hormone (LH)) the gonads synthesize and secrete sex steroids causing feedback on pituitary and hypothalamus (modified from Heikkilä 2002).

Neurosecretory cells of the hypothalamus regulate the production and secretion of gonadotropin-releasing hormone (GnRH) (Zohar et al. 2010), when integrating exogenous and endogenous stimuli that influence the central nervous system. GnRH stimulates the release of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary. LH and FSH, in turn, stimulate the gonads to produce and release the sex steroids, androgens, estrogens and progetstogens. These sex steroids then affect target cells and, moreover, regulate homeostasis through negative feedback on hypothalamus and pituitary.

The GnRH family has 16 structurally distinct variants, of which at least two are found in each species of vertebrates (Powell et al. 1995; Somoza et al. 2002). In the anuran brain, two distinct populations of GnRH forms were discovered yet (Jokura and Urano 1986; Wilczynski and Northcutt 1994). Neurons of the first population, the mammalian luteinizing hormone releasing hormone (LHRH) form, were suggested to account for GnRH release into the pituitary. The second GnRH population, which is characterized by the chicken II (CII) form of GnRH, was suggested to act as neuromodulator within the central nervous system (Wilczynski and Northcutt 1994). An even greater diversity in the number of GnRH forms has been found in fishes, where different species express various forms of GnRH (Powell et al. 1994, 1995; Somoza et al. 2002; Okubo et al. 2008), some of them regulating the production and secretion of LH and FSH from the pituitary.

The heterodimeric glycoprotein-hormones LH and FSH and their corresponding receptors are present in all vertebrates other than squamates (Licht and Papkoff 1974; Papkoff et al. 1976; Levavi-Sivan et al. 2010). They play an important role in processes of cell differentiation, proliferation and steroidogenesis (Polzonetti-Magni et al. 1998; Lubzens et al. 2010). While LH was shown to promote androgen and progesterone (P4) secretion (Polzonetti-Magni et al. 1998; Norris 2006), final oocyte maturation and ovulation (Norris 2006; Lubzens et al. 2010), as well as testicular maturation and spermiation (Norris 2006), FSH is considered to stimulate estrogen secretion and lactation (Mita et al. 1982; Herman 1992; Polzonetti-Magni et al. 1998), early development of the ovarian follicle and spermatogenesis in the testis (Norris 2006; Lubzens et al. 2010). Nevertheless, it is noteworthy that gonadotropic effects are assumed to be mainly indirect via secretion of estrogens, androgens and progetstogens (Licht 1979; Kloas et al. 2009).

Besides controlling physiological functions, such as metabolism (Kirschener et al. 1982; Mauvais-Jarvis 2011) and immune system functions (Grossman 1985; DaSilva 1999; Cutolo et al. 2004), androgens, estrogens and progetstogens (Fig. 2) are crucial for reproductive processes. They stimulate final gamete maturation, as well as ovulation and spermiation (Polzonetti-Magni et al. 1998; Norris 2006) and induce vitellogenesis (Ryffel et al. 1977; Lubzens et al. 2010). Moreover, sex steroids are involved in expression of secondary sexual characteristics (Borg et al. 1993; Harvey and Propper 1997) and reproductive behaviors, such as mating and parental care

(Wetzel and Kelley 1983; Harvey and Propper 1997; Munakata and Kobayashi 2010). The androgens testosterone (T) and dihydrotestosterone (DHT) can be found in anurans, as well as in all higher vertebrates. In urodeles and fish, however, the predominant androgen is 11-ketotestosterone (Tinsley and Kobel 1996; Kloas et al. 2009).

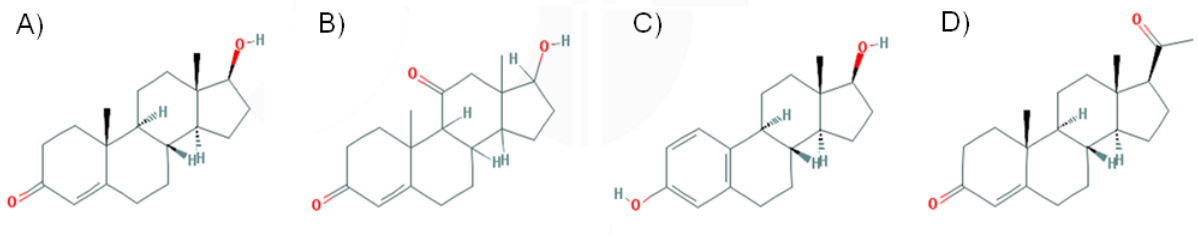

Fig. 2: Structural formulas for (A) the androgen testosterone, (B) the androgen 11-ketotestosterone, (C) the estrogen 17β-estradiol and (D) the natural progesterone.

In principle, sex steroids act via nuclear receptors, directly activating transcriptional cascades (Aranda and Pascual 2001; Björnström and Sjöberg 2005), however, they can also bind to membrane-associated steroid receptors, initiating non-genomic steroid actions. They also can activate non-genomic protein-protein interactions with DNA-binding transcription factors, enabling further genes to be regulated (Björnström and Sjöberg 2005; Watson et al. 2011). Furthermore, sex steroids regulate gonadotropin production and secretion via negative feedback on hypothalamus and pituitary and thereby maintaining homeostasis of the organism (McEwen et al. 1978; McCreery and Licht 1984; Kloas and Lutz 2006). Homeostasis is further preserved by aromatase action, the enzyme that converts aromatizable androgens into estrogens (Lofts 1974; McNatty et al. 1979a, 1979b; Callard et al. 2001; Levavi-Sivan et al. 2006). The sensitivity of hypothalamus and pituitary concerning negative feedback of sex steroids is usually dependent on seasonal fluctuations in HPG activity (Tilbrook et al. 1999; Burmeister and Wilczynski 2005).

1.3 Effects of (anti)androgenic and (anti)estrogenic endocrine disrupting compounds on reproductive biology of aquatic vertebrates

The HPG axis offers multiple targets for interferences with EDCs with (anti)estrogenic and (anti)androgenic MOAs. Nevertheless, most of the effects of such EDCs are assumed to be mediated directly via sex steroid receptors (agonistic or antagonistic) or indirectly by altering synthesis and bioavailability of sex steroids within the organism (Kloas et al. 2009). In developing individuals, early development and the phase of sexual differentiation are the most important stages in which EDCs

negatively impact reproductive biology (Oka et al. 2006; Cevasco et al. 2008; Kloas et al. 2009), whereas in adults, (anti)androgenic and (anti)estrogenic EDCs adversely affect preservation and maintenance of reproductive functions (Zala and Penn 2004; Scott and Sloman 2004; Kloas et al. 2009; Saaristo et al. 2009).

Effects of androgenic endocrine disrupting compounds

Androgenic EDCs interfere with androgen signaling pathways by agonistic binding to androgen receptors (AR) and expression of androgen-regulated genes, as well as by promoting negative androgen feedback to pituitary. Effects of androgenic EDCs have been demonstrated, e.g. in juvenile fish (Pifferrer et al. 1991, 1993; Bogers et al. 2006) and anurans (Bögi et al. 2002; Kloas et al. 2002), where an increase in male phenotypes was observed after androgenic exposure. However, differences in masculinizing potency were noticed between aromatizable and non-aromatizable androgenic EDCs, with non-aromatizable androgens being much more effective (Pifferrer et al. 1991, 1993; Bögi et al. 2002). Moreover, histopathological modifications like thyroid follicular hypertrophy, germ cell necrosis, as well as female ovarian atresia were detected after exposure of juvenile fish (Hahlbeck et al. 2004 a and b; León et al. 2007).

In adult male frogs, exposure to the androgenic EDCs methyltestosterone (MT) or DHT led to lower circulating LH and decreased pituitary and brain LH concentrations (Tsai et al. 2005; Urbatzka et al. 2006b) resulting in disrupted spermatogenesis (Tsai et al. 2005). In adult female frogs, oocyte atresia and spermatogenic nests were observed after treatment with methyldihydrotestosterone (MDHT) (Cevasco et al. 2008). In fish, on the other hand, MT and MDHT exposure were found to stimulate Sertoli cells and spermatogenesis in males, whereas female vitellogenesis, as well as oogenesis and ovarian development were suppressed (Van der Ven et al. 2003; Chikae et al. 2004; Bogers et al. 2006). A significant decrease in fecundity was observed in fathead minnow pairs exposed to MDHT (Bogers 2006) and in three-spined sticklebacks, androgen exposure at low concentrations was shown to induce the spiggin protein in kidney, which is used as glue in nest building (Hahlbeck et al. 2004b; Jolly et al. 2006; Björkblom et al. 2007).

Effects of antiandrogenic endocrine disrupting compounds

EDCs with antiandrogenic MOA can interfere with reproductive biology via two mechanisms. They can bind to AR, thereby block androgen access, which is necessary for stabilizing the naturally rapidly degrading AR, and change receptor conformation in such a way, that DNA binding is prevented and the AR remains inactivated within the cell or degrades (Kuil 1990; Kelce and Wilson 1997). However, antiandrogen binding to the AR can also actively induce DNA binding but fail to initiate transcription (Kuil 1990; Kelce et al. 1997). Either way, by blocking AR, antiandrogens reduce the action of natural androgens, simulating lower androgen levels and hampering negative androgen feedback to pituitary (Urbatzka et al. 2006b).

Exposure of tadpoles and fish larvae to antiandrogenic EDCs resulted in feminization (Bayley et al. 2002; Bögi et al. 2002; Kloas 2002), reduced growth (Makynen et al. 2000; León et al. 2007) and malformation of male and female gonads (Bayley et al. 2002; Bögi et al. 2002; Kiparissis et al. 2003). Exposure of juvenile fish to the antiandrogen vinclozolin (VIN) also resulted in reduction of secondary sexual characteristics, reduced sperm count and suppressed courtship behavior (Bayley et al. 2002), as well as reduced fertility (Bayley et al. 2003) when fish matured.

A reduced number of ejaculated sperm cells, a reduction of secondary sexual characteristics and suppressed courtship behavior were also noticed, when adult fish were exposed to the antiandrogens VIN or flutamide (FLU) (Martinović et al. 2007; Sebire et al. 2008; Baatrup and Junge 2001). Furthermore, antiandrogenic exposure led to increased vitellogenin (Vtg) levels (Chikae et al. 2004; Jensen et al. 2004; Panter et al. 2004) and reduced gonadal condition and fecundity of female but not male fish (Makynen et al. 2000; Martinovic et al. 2007), whereas higher estradiol levels and lower levels of spiggin were only observed in males (Makynen et al. 2000; Jensen et al. 2004), along with spermatocyte degeneration, necrosis (Jensen et al. 2004) and the occurrence of oocytes in testes (León et al. 2007). In male frogs (*Xenopus laevis*), FLU exposure resulted in only minor effects on testes: no oocytes were found, however, the average tubule diameter was reduced (Cevasco et al. 2008). Nevertheless, FLU treatment increased atretic oocytes and decreased the percentage of mature oocytes in female frogs (Cevasco et al. 2008). Moreover, a suppressed mate calling behavior was noticed in male *X. laevis* exposed to FLU for 3 consecutive days (Behrends et al. 2010).

Effects of estrogenic endocrine disrupting compounds

Xenoestrogens mimic the effects of estrogens e.g. by activating the expression of estrogen-responsive genes (Brosens and Parker 2003) and by interfering with gonadotropin production and secretion via feedback on hypothalamus and pituitary (McEwen et al. 1978; McCreery and Licht 1984; Kloas and Lutz 2006). Exposure of embryos and juvenile aquatic vertebrates to the natural 17β-estradiol (E2) or the estrogenic EDC 17α-ethinylestradiol (EE2) was shown to cause abnormal gonadal function and morphology, as well as feminization (Kloas et al. 1999, 2002; Brion et al. 2004; Fenske et al. 2004; Xu et al. 2008; Oka et al. 2006). Some of the feminizing effects were suggested to be due to upregulation of aromatase gene expression in testes and brain (Scholz and Gutzeit 2000; Kuhl et al. 2005), the enzyme responsible for the conversion of androgens to estrogens. In addition, hepatic Vtg induction, suppressed organogenesis and even embryo death were observed in juvenile fish and amphibians of both sexes (Nishimura et al. 1997; Bevan et al. 2003; Brion et al. 2004).

As in juveniles, hepatic Vtg induction was also demonstrated in estrogen exposed adult aquatic vertebrates (Islinger et al. 2003; Brion et al. 2004; Pawlowski et al. 2004; Rasmussen et al. 2005; Urbatzka et al. 2007; Saaristo et al. 2010a and b). Moreover, in male amphibians small oocytes occurred within testes after exposure to EE2 (Cevasco et al. 2008). In fish and amphibians, secondary sexual characteristics, such as uro-genital papillae (Brion et al. 2004) and nuptial pads (Hayes et al. 2010), respectively, were shown to be modified by estrogenic exposure. Impairments of reproductive behaviors of fishes after short- and long-term exposure to environmentally relevant concentrations of E2 and EE2, respectively, were detected and assumed to result in a selection against exposed fish (Bayley 1999; Xu et al. 2008; Colman et al. 2009; Saaristo et al. 2009, 2010a and b; Partridge et al. 2011).

Effects of antiestrogenic endocrine disrupting compounds

On the basis of the mechanism of action, antiestrogens form two groups: direct-acting and indirect-acting antiestrogens. By competitively inhibiting estrogen receptors (ER), antiestrogens directly interfere with estrogen action and negative estrogenic feedback to pituitary, while antiestrogens that affect the normal turnover of estrogens, e.g. via aromatase inhibition, are considered to act indirectly (U.S. EPA 2000; Sun et al. 2011).

In fish, exposure of genetic female larvae to the antiestrogen tamoxifen (TAM) led to masculinization via downregulation of aromatase mRNA expression (Kitano et al. 2007). A second effect of this antiestrogenic exposure was demonstrated to be the upregulation of Müllerian inhibiting substance (MIS) mRNA expression (Kitano et al. 2007). MIS is a substance that was previously shown to actively cause the regression of the Müllerian ducts, preventing the process of female reproductive organ development (Behringer at al. 1994). The upregulation of MIS resulted in phenotypic males and undifferentiated fish (Andersen et al. 2004; Kitano et al. 2007). In amphibians, sex differentiation was also shown to be affected by TAM exposure; however, exposure resulted in neutralization and underdeveloped, non-functioning gonads (Bögi et al. 2002; Kloas et al. 2002). In hatchling alligators, intramuscularly antiestrogen injections blocked the estradiol-induced oviductal hypertrophy and the estradiol-induced hepatic Vtg secretion (Lance and Bogart 1990). A suppressed hepatic Vtg induction was also demonstrated in juvenile fathead minnows after a 14-day exposure to the antiestrogen ZM189154 (Panter et al. 2002).

In adult fish, exposure to xenoestrogens is known to increase plasma Vtg levels (Leanos-Castaneda et al. 2004; Rasmussen et al. 2005; Sun et al. 2009), to result in malformation of testes and oocytes (Rasmussen et al. 2005) and to impair reproductive performance (Sun et al. 2009). Co-treatment with antiestrogens was demonstrated to abolish estrogen-induced Vtg induction (Leanos-Castaneda et al. 2004; Rasmussen et al. 2005; Sun et al. 2009, 2011), however, estrogenic effects on the testes could only be suppressed in part (Rasmussen et al. 2005; Sun et al. 2009) or even became more severe (Sun et al. 2009). In frogs, the stimulating potency of estrogens on regressed oviducts of ovariectomized females was suppressed by simultaneous treatment with the antiestrogen ICI 46474 (Rastogi and Chieffi 1974). Sole antiestrogenic exposure was shown to decrease Vtg mRNA expression in female frogs (Urbatzka et al. 2007). Moreover, in female but not in male frogs, aromatase mRNA expression decreased (Massari et al. 2010), whereas plasma E2 levels, as well as mRNA expression of LH and FSH increased due to TAM exposure (Urbatzka et al. 2006b, 2007), inducing defects like spermatogenic nests and oocyte atresia (Cevasco et al. 2008). Urbatzka and colleagues (2007) suggested that in *X. laevis*, antiestrogens interfere with the estrogenic feedback on hypothalamus and pituitary and because females have high endogenous estrogen levels, antiestrogenic treatment can easily alter these levels in a considerable amount, resulting in a

modified estrogenic feedback. Alterations of the naturally low endogenous estrogen levels in males, however, might not be severe enough to affect estrogen feedback (Urbatzka et al. 2007).

1.4 The model species *Xenopus laevis*

The South African clawed frog, *X. laevis* (Anura: Pipidae; Fig. 3), was first described at the beginning of the 19th century.

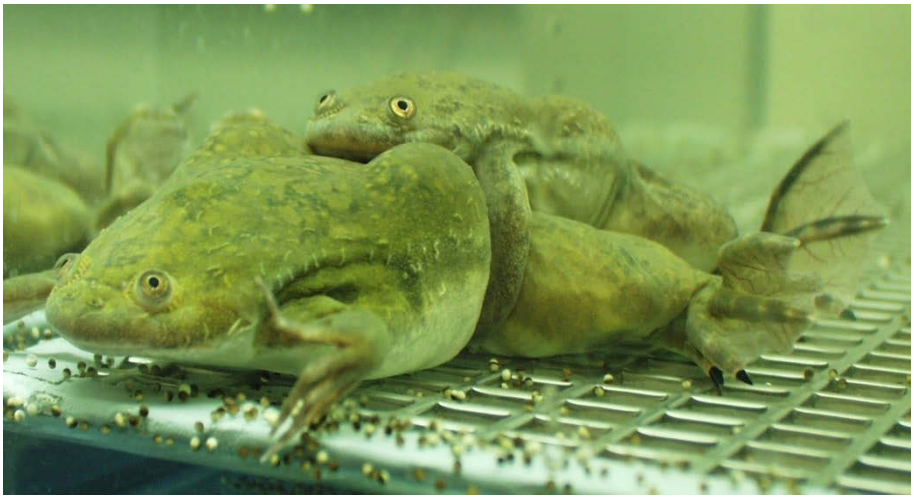

Fig. 3: Picture of two adult South African clawed frogs, *Xenopus laevis* (photo by Dr. Ilka Lutz). The smaller male (back) is clasping the bigger female (front) until oviposition.

The indigenous range of this species is the sub-Saharan Africa, from Sudan to South Africa (Kobel et al. 1996; Tinsley et al. 1996; Channing 2001). In addition, *X. laevis* also occurs non-indigenously in several parts of the U.S., Mexico, Chile, Europe and Japan (Tinsley and Kobel 1996), certainly due to escapes of this model organism, extensively used in scientific research as well as to intentional introduction of this species (Tinsley et al. 1996). *X. laevis* is a primarily aquatic vertebrate which can easily disperse overland in order to occupy new habitats (Tinsley et al. 1996; Channing 2001). It can survive in various water conditions, e.g. up to 40% seawater, a temperature range between 2°C and 35°C and a pH ranging from 5 to 9, which makes this species a preferable model organism with easy husbandry conditions. Streams and dark and turbid ponds and ditches are the main natural habitats of *X. laevis*, whereas this carnivorous species avoids larger rivers and areas inhabiting large predators (Tinsley and Kobel 1996). After hatching of larvae, two to three days after oviposition, *X. laevis* tadpoles feed suspended algae until metamorphosis. Time to metamorphosis depends on water temperature and the availability of food but usually metamorphosis occurs within 75 days after hatching (Coady et al. 2005).

Post-metamorphic South African clawed frogs become mature within 6 - 8 months and can survive up to 20 years (Tinsley and Kobel 1996).

Mate calling behavior of male Xenopus laevis and its endocrine regulation

Because the preferred habitats of *X. laevis* are dark, turbid ponds, males rely on underwater acoustic cues to broadcast sexual arousal and location (Kelley and Tobias 1999). These vocalizations encompass several different call types (Tobias et al. 1998b). Each call type is composed of repetitive trills, consisting of trains of click sounds, brief and noisy sound elements in a frequency range between 1 kHz and 3 kHz. Clicks are produced by contractions of laryngeal muscles (Yager 1982; Fig. 4) innervated by neurons of cranial nerve nucleus IX-X within the vocal pathway, a defined neural circuit in the central nervous system (CNS).

Fig. 4: Larynx (vocal organ) of male *Xenopus laevis*. The major structural components of the larynx include hyaline cartilage (hc) which forms the cartilaginous box, arytenoids cartilage which forms the sound producing arytenoid disks (ad), surrounded by elastic cartilage (ec). Sounds are produced when the laryngeal bipennate muscles contract and, acting via tendonous insertions onto the arytenoids disks, pull the disks apart (modified from Fischer and Kelley 1991; Kelley and Tobias 1999).

Vocalizations of *X. laevis* are highly stereotyped and thus different call types are distinguishable from each other with respect to spectral and temporal parameters (Tobias et al. 1998b, 2004). To attract females and to keep away opponents, males produce advertisement calls (AC), which consist of alternating slow (35 Hz) and fast (70 Hz) trills that have a peak frequency between 1.8 kHz and 2.3 kHz (Wetzel and Kelley 1983; Fig. 5).

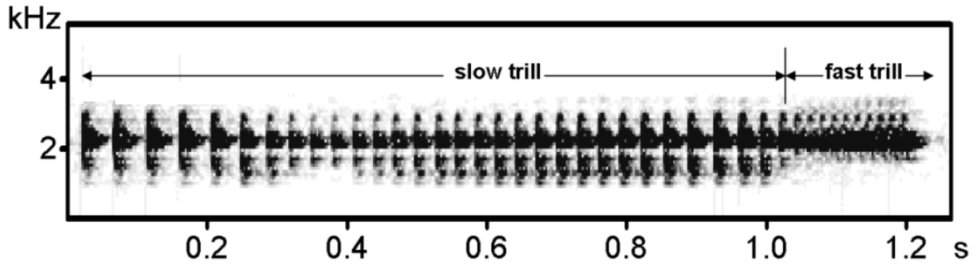

Fig. 5: Spectrogram of an advertisement call of a male *Xenopus laevis*. Left: slow trill part, right: fast trill part with overlapping clicks.

Females have fairly short periods of sexual receptivity (Kelley 1996), during which they are attracted to and sexually stimulated by male AC (Picker 1980, 1983; Tobias et al. 1998b). In response to male AC, receptive females produce rapping calls, irregular rapid trills with a peak frequency of around 1.2 kHz, and swim towards the calling male (positive phonotaxis). Is a rapping female in close proximity, the sexually aroused male swims to the female and clasps her while uttering answer and amplectant calls (Picker 1980; Tobias et al. 1998b; Tobias and Kelley 1999; Kelley 2004). Amplectant calls are slow two- and three click calls with 1 s pauses between calls, while answer calls look similar to advertisement calls; however, the fast trills of answer calls last longer, while the short trill proportions are shorter than within AC (Picker 1980; Kelley and Tobias 1999). Eventually, the clasped receptive female oviposits up to ca. 1000 eggs, which are then fertilized by the clasping male (Tinsley and Kobel 1996).

Is an unreceptive female clasped by a male, the female utters female ticking calls, slow clicks with a peak frequency of 1.2 kHz, which are also called release calls (Kelley and Tobias 1999). Males can also produce ticking calls, e.g. when clasped by another male (Tobias et al. 2004). Male ticking consist of slow monotonous clicks (4 Hz) with a peak frequency of 1.7 – 2.8 kHz mostly uttered within male-male interactions (Tobias et al. 2004; Fig. 6).

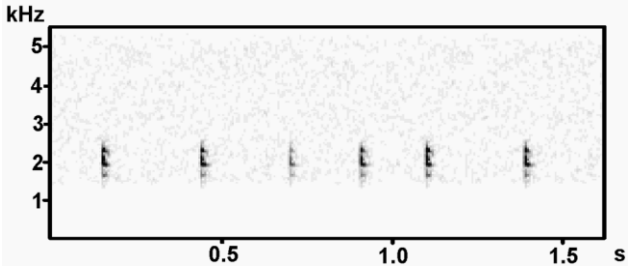

Fig. 6: Spectrogram of the call type ticking.

Males utter further call types that accompany e.g. male-male interactions: chirping and growling. Chirping is produced by a male which is clasping another male. It is composed of a series of fast trills (70 Hz) that are separated by a 0.2 – 0.4 s interval and has a peak frequency around 2 kHz (Kelley and Tobias 1999, Fig. 7).

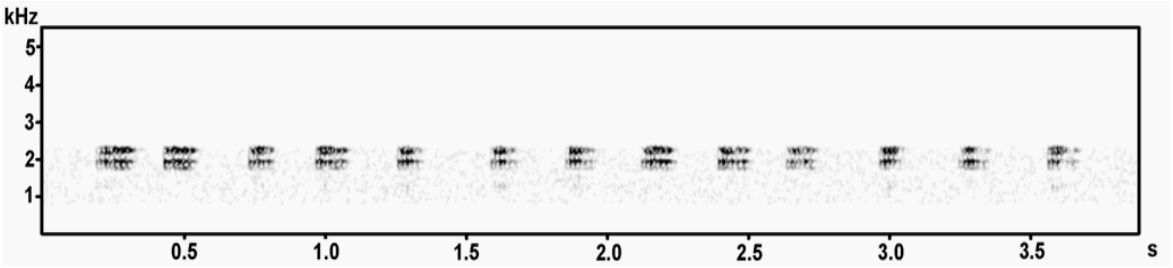

Fig. 7: Spectrogram of the call type chirping.

Growling, on the other hand, is a male release call, produced when the male is clasped by another male. It is composed of rapid, low pitched (~1 kHz) trills (Kelley and Tobias 1999; Fig. 8).

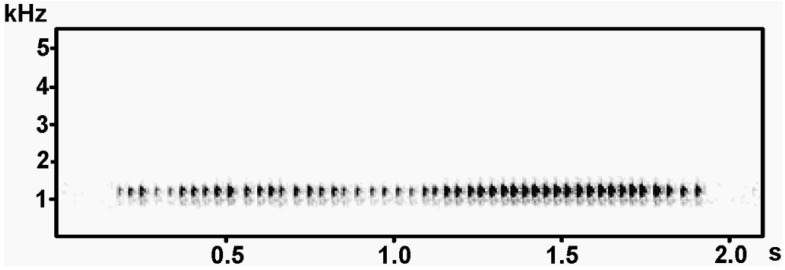

Fig. 8: Spectrogram of the call type growling.

Male mate calling behavior of *X. laevis* is controlled by the HPG axis (Segil et al. 1987; Kelley et al. 1989; Tinsley and Kobel 1996; Kelley and Tobias 1999). Particularly androgens are suggested to control this behavior. Environmentally relevant concentrations of the antiandrogen FLU for example were shown to decrease male mate calling activity of *X. laevis* (Behrends et al. 2010). Moreover, male mate calling behavior is abolished by castration and cannot be restored by gonadotropin injections (Kelley and Pfaff 1976) but it can be restored by treatment with exogenous androgens (Wetzel and Kelley 1983). Although the nonaromatizable androgen DHT was shown to be particularly effective, androgen treatment does not restore advertisement calling to precastrate levels (Wetzel and Kelley 1983). Hence, sex steroids are necessary for the production of male mate calling, however, further (non-steroidal) hormones, like GnRH, gonadotropins and prostaglandins were shown

to act in conjunction with the sex steroids (Kelley 1982; Wetzel and Kelley 1983; Weintraub et al. 1985; Taylor and Boyd 1991).

The larynx of *X. laevis* was shown to be the effector organ for the male-specific vocalizations. Laryngeal muscle fibers of male *X. laevis* express high levels of AR (Kelley et al. 1975; Kelley 1981; Sassoon and Kelley 1986; Segil et al. 1987) and have been shown to be a direct target of androgen action (Kelley 1980; Gorlick and Kelley 1987; Kelley and Tobias 1999). Another effector is the vocal pathway. The vocal pathway (Fig. 9) includes the ventral striatum (VST) and the preoptic area, the inferior reticular formation (Ri), brainstem nuclei, the dorsal tegmental area of the medulla (DTAM), the dorsal nucleus raphe (rRpd), and neurons in cranial nerve nucleus IX-X that innervate the larynx (Wetzel et al. 1985, Emerson and Boyd 1999; Brahic and Kelley 2003). DTAM, rRpd, Ri, and the nerve nucleus IX-X are all responsible for generating patterned vocal activity in the central vocal-motor pathway of *X. laevis* (Brahic and Kelley 2003). Neurons throughout this pathway express ARs and therefore could be direct targets of androgen action (Kelley et al. 1975; Kelley 1981; Perez et al. 1996).

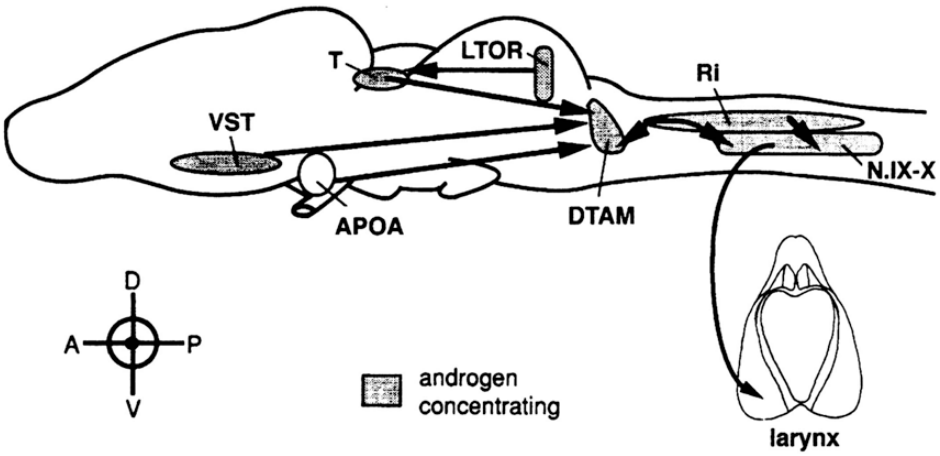

Fig. 9: The vocal pathway of *Xenopus laevis*. Motor neurons in the cranial nerve nucleus IX-X (N. IX-X) project via the laryngeal nerve to the bipennate muscles. N IX-X contains motor neurons and interneurons and receives projections from adjacent inferior reticular formation (Ri) and a superior reticular nucleus, the dorsal tegmental area of the medulla (DTAM); in males this connection is reciprocal. DTAM receives input from the dorsal diencephalon (auditory thalamus: T) and ventral telencephalon (APOA: anterior preoptic area; VST: ventral striatum). The laminar nucleus of the torus semicircularis (LTOR) provides auditory input to the thalamus (from Kelley and Tobias 1999).

Especially the VST was found to be a strong candidate for acoustic pattern modulation of vocal signaling in social interactions of male and female *X. laevis* (Yang and Kelley 2008). It expresses very high levels of gonadotropin receptors (Morell et al. 1975; Yang et al. 2007). Thus, in addition to androgens, gonadotropins,

such as LH, may also directly influence male vocalizations via the vocal pathway. Moreover, external factors, such as water temperature (Kalk 1969), rainfall (Picker 1983), and the presence of algae (Bles 1906; Savage 1965) were also demonstrated to influence natural spawning and male mate calling activity of *X. laevis.*

In the laboratory, injections of human chorionic gonadotropin (hCG) stimulate male and female *X. laevis* to display high levels of sexual behavior (Picker 1980; Kelley and Pfaff 1976; Kelley 1982). HCG stimulates females' receptivity (Kelley 1982) and the production of *rapping* calls (Tobias et al. 1998b); it promotes females' phonotactic response (Picker 1980) and results in ovulation and oviposition (Kelley 1982). HCG injected males show enhanced mate calling and clasping behavior (Kelley and Pfaff 1976; Kelley and Tobias 1999).

1.5 Aim of the study and model substances

Although EDCs are suspected to markedly contribute to the worldwide decline of amphibian populations (Carey and Bryant 1995), relatively little is known about the potential effects of EDCs in amphibians. Only recently *X. laevis* became a well established model organism for the study of EDC effects, especially for the assessment of (anti)androgenic and (anti)estrogenic EDCs affecting reproductive biology *in vitro* and *in vivo* (Kloas et al. 1999, 2009; Bögi et al. 2002; Levy et al. 2004). Nevertheless, to date most of the existing biomarkers for the assessment of (anti)androgenic and (anti)estrogenic EDCs using *X. laevis* as model species are invasive techniques. Animals are exposed to EDCs during larval stages, which generally results in permanent and irreversible impacts, or, like in most cases, experimental animals need to be sacrificed in the analyzing processes (Kloas et al. 2009). Non-invasive techniques, e.g. short-term exposure of adult frogs leading to reversible effects, do not exist yet.

Since the male mate calling behavior of *X. laevis* was shown to depend on sex steroids and gonadotropins (Morell et al. 1975; Wetzel and Kelley 1983; Yang et al. 2007), this behavior might be an appropriate endpoint for the assessment of (anti)estrogenic and (anti)androgenic EDCs. Behrends et al. (2010) demonstrated already that the total vocal output of male *X. laevis* decreases, when individuals are exposed to environmentally relevant concentrations of the antiandrogen FLU. However, further studies testing the suitability of this endpoint as biomarker for the assessment of (anti)androgenic and (anti)estrogenic EDCs are lacking. Hence, this

study was performed to examine whether environmentally relevant concentrations of EDCs with (anti)androgenic and (anti)estrogenic MOAs affect the androgen-controlled male mate calling behavior of *X. laevis* and whether this endpoint might be used as biomarker for the assessment of such EDCs.

Model substances with (anti)androgenic and (anti)estrogenic modes of action

To examine whether environmentally relevant concentrations of EDCs with (anti)androgenic and (anti)estrogenic MOAs affect the male mate calling behavior of *X. laevis*, various model substances were used. MDHT and VIN were used to cover androgenic and antiandrogenic MOAs, respectively, while EE2 was used as estrogenic EDC. Fulvestrant (ICI), as well as TAM served as antiestrogenic model substances.

Androgenic methyldihydrotestosterone

MDHT (Fig. 10) is a widely used model compound for the assessment of androgenic EDCs (Van der Ven et al. 2003; Urbatzka et al. 2006a, 2006b, 2007; Cevasco et al. 2008; Hoffmann et al. 2008), because it binds with high affinity to nuclear androgen receptors (Piferrer et al. 1993; Sperry and Thomas 2000; Thomas et al. 2002). Nevertheless, to my knowledge no studies exist in which MDHT concentrations in water sources are determined, although alarming levels of androgenic activity, in general, have been found in effluents (~ 600 ng/L dihydrotestosterone (DHT) equivalents) (Jenkins et al. 2001; Kirk et al. 2002; Liu et al. 2011; Thomas et al. 2002) and surface waters (~ 55 ng/L DHT equivalents) (Liu et al. 2011). In sediments samples from the Italian river Lambro, MDHT equivalents of up to 40 µg/kg sediment were found (Urbatzka et al. 2007).

In fish, implants of nonaromatizable androgens (DHT) were shown to be effective in restoring courtship behavior in castrated males (Andreoletti et al.1983), as it was demonstrated for castrated male *X. laevis* in which nonaromatizable androgen implants (DHT) reinstated male advertisement calling (Wetzel and Kelley 1983). However, advertisement calling of *X. laevis* was not affected by low doses of DHT (Wetzel and Kelley 1983), thus it remains unclear whether environmentally relevant concentrations of nonaromatizable androgenic EDCs affect male amphibian courtship

behavior. Furthermore, to the best of my knowledge there are no studies investigating behavioral effects of MDHT in aquatic vertebrate species.

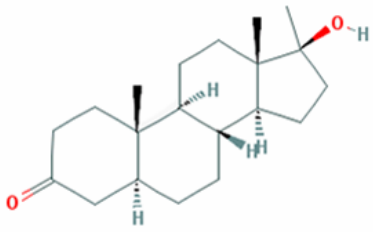

Fig. 10: Structural formula of 17α-methyldihydrotestosterone (MDHT).

Antiandrogenic vinclozolin

The mass-produced fungicide VIN has been shown to be antiandrogenic (Kang et al. 2004). Although the use of VIN was forbidden in Germany in 2001, VIN is still commonly used against some fungi on fruits, vegetables, and wine grapes across the United States of America and Europe, illicitly including Germany (Spencer 1982; Laws et al. 1996; U.S. EPA 2000; Greenpeace 2004, 2005). Its consumption is considered to be several tons per year (Readman et al. 1997; Steeger and Garber 2009). VIN and its two metabolites, M1 and M2, (Kelce et al. 1994a) have the capability to relocate from treatment sites to non-target areas by runoff and leaching (Steeger and Garber 2009). Accordingly, in surface waters, VIN has been detected at concentrations of up to 0.5 µg/L (Gülden et al. 1997; Readman et al. 1997; El-Shahat et al. 2003), and even in drinking water, its maximum detected concentration was 0.1 µg/L (Iwan 1988). Besides its use in agriculture, VIN is also used as a model substance for antiandrogenic modes of action (Ottinger et al. 2001; Kubota et al. 2003; Loutchanwoot et al. 2008). VIN and its metabolites (Fig. 11), M1 and M2, competitively inhibit androgen binding to the AR by fitting into the hormone binding domain (Kelce and Wilson 1997). Once bound to the AR, these compounds are imported to the nucleus, where they inhibit the expression of AR-dependent genes by inhibiting the ability of the androgen-bound AR to bind androgen response element DNA (Kelce et al. 1994b, 1997; Wong et al. 1995).

Studies in rats and birds demonstrate that VIN treatment can alter hormonally regulated behaviors (Hotchkiss et al. 2003; Satre et al. 2009), however, evidence for VIN affecting amphibian mating behavior is lacking.

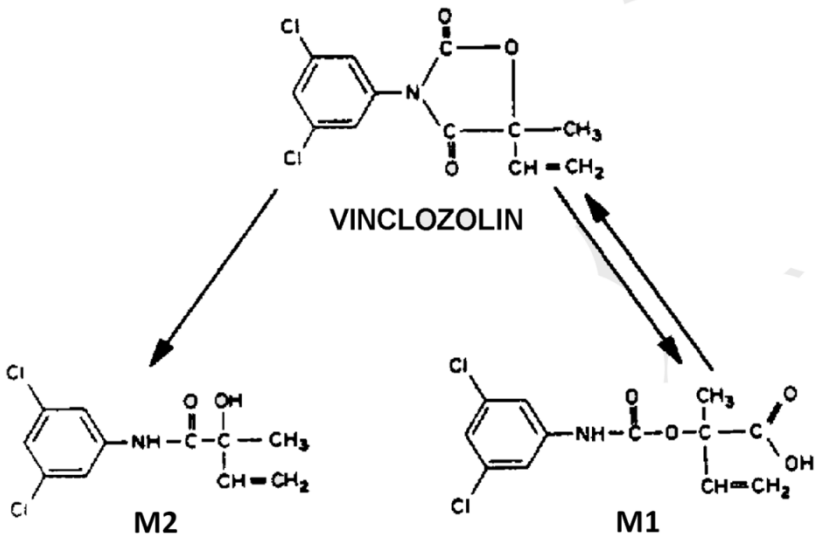

Fig. 11: Degradation pathway proposed by Szeto et al. (1998) for the hydrolysis of vinclozolin (VIN) leading reversibly to the formation of 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and irreversibly to the formation of 3',5'-dichloro-2-hydroxy-2-methylbut-3-enan-ilide (M2) (modified from Kelce et al. 1994a).

Estrogenic 17α-ethinylestradiol

The estrogen EE2 (Fig. 12) is a main component of many classical contraceptives. In the EU, only around 50 kg of EE2 are produced each year but the prescription rate of this drug is very high (Sanderson et al. 2004). In the US it is assumed that 88 kg EE2 per year are used (Arcand-Hoy et al. 1998).

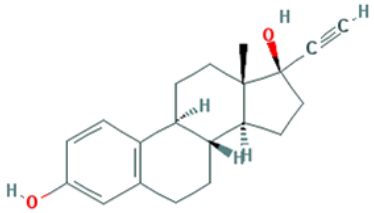

Fig. 12: Structural formula of 17α-ethinylestradiol (EE2).

EE2 inhibits ovulation (Emperaire and Greenblatt 1969; Greenblatt et al 1974) by suppressing FSH secretion and altering structures of the endometrium (van Heusden and Feuser 2002; Prasad 2010). Unfortunately, EE2 is also a compound of high concern, because it is excreted unmetabolized through feces and urine (Orme et al. 1983; Braun et al. 2003) and enters the environment via wastewater effluents (Jones et al. 2001). It displays high estrogenic activity even at extremely low concentrations (Purdom et al. 1994; Jobling et al. 1998) and has been detected in effluents (Stumpf et al. 1996; Belfroid et al. 1999; Ternes et al. 1999) and in surface waters (Desbrow et al. 1998; Belfroid et al. 1999; Shen et al. 2001) at concentrations ranging from 7 –

64 ng/L and from 0.1 – 30 ng/L, respectively. Moreover, EE2 could even be detected in drinking water at concentrations of up to 1.4 ng/L (Adler et al. 2001; Kuch and Ballschmiter 2001).

EE2 was shown to impair reproductive behaviors of fishes (Bjerselius et al. 2001; Xu et al. 2008; Colman et al. 2009; Saaristo et al. 2009; Partridge et al. 2010), however evidence for EE2 affecting amphibian mating behavior is lacking.

Antiestrogenic tamoxifen and fulvestrant

The non-steroidal estrogen antagonist TAM (Fig. 13 a) is a pharmaceutical used to treat advanced breast cancer (Heuson 1976; Jackson et al. 1991). It was proven to reduce the risk of estrogen receptor-positive, but not estrogen receptor-negative, tumor recurrence and to prolong survival when administered as postoperative adjuvant therapy (Fisher et al. 1986; Fisher et al. 1989; Fisher et al. 1998). By selectively modulating ER, TAM can exhibit different mechanisms of action in different tissues (Shou et al. 2004). It inhibits transcriptional activity of ER in breast tissue (Shou et al. 2004, MacGregor and Jordan 1998) and exhibits estrogen-like activity in bone and uterine tissue (Webb et al. 1995; MacGregor and Jordan 1998; Bentrem et al. 2001), which can be masked at high doses (Horowitz and McGuire1978). These different mechanisms of action are assumed to result from interactions between TAM and various proteins involved in the transcription of estrogen-responsive genes (MacGregor and Jordan 1998).

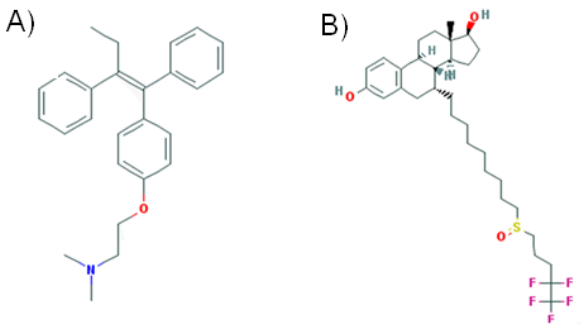

Fig. 13: Structural formulas of (A) tamoxifen (TAM) and (B) fulvestrant (ICI).

The E2 analogue Fulvestrant (ICI 182780; ICI; Fig. 13 b), on the other hand, is a pure estrogen antagonist with no estrogenic properties (MacGregor and Jordan 1998). Due to the lack of estrogen-like activity, ICI was shown to suppress tumor cell growth, delay tumorigenesis and reduce the expression of estrogen-regulated genes to a greater extent than TAM (Osborne et al. 1995). However, eventually most tumors

showed resistance to ICI and tumor growth became independent from estrogen presence (Osborne et al. 1995). Having a greater ER affinity than TAM (Wakeling and Bowler 1988), ICI competitively inhibits E2 binding to the ER. The steroidal structure of ICI, containing a bulky side chain (Pike et al. 2001), leads to conformational changes in the ligand binding domain (Wu et al. 2005), impairs receptor dimerization (Fawell et al 1990; Dauvois et al. 1993) and thereby inactivates transcription (Osborne et al. 2004). Moreover, ICI-ER complexes are highly instable. Thus, ER down-regulation occurs due to ER protein degradation (Nicholson et al. 1995; Long and Nephew 2006), resulting in a complete inhibition of estrogen signaling through ER (Osborne et al. 1995; Wakeling 1995; Wardley, 2002).

TAM and ICI can enter waste- and surface waters by being excreted by humans after ingestion and sewage treatment works (STW) often fail in removing those substances. Hence, those EDCs can be found at high concentrations in already treated effluents (Ternes et al. 1999; Hilton et al. 2003). TAM was detected in effluents in the UK at concentrations ranging from 20 – 40 ng/L (Hilton et al. 2003) and in UK estuaries 13 – 200 ng/L TAM were found (Roberts and Thomas 2006). In the EU, ICI is licensed since 2004 and in the US it is on the market since 2002; ICI was shown to be more effective in reducing cell turnover index compared to TAM (Bundred et al. 2002), thus its use increased over the last years (Doloresco 2011). Nevertheless, studies investigating the amount of ICI that can be found in the environment are lacking.

Study aim

The aim of the study was to determine whether environmentally relevant concentrations of (anti)androgenic and (anti)estrogenic EDCs affect the male mate calling behavior of *X. laevis* and whether this endpoint might be used as biomarker for the assessment of such EDCs. In addition, for a marked improvement of the basic methodology introduced by Behrends et al. (2010), a more detailed analysis of call types was developed to allow for improved sensitivity to identify specific MOAs of EDCs (androgenic, antiandrogenic, estrogenic and antiestrogenic) as well as to determine levels of sexual arousal of exposed males. Additional tests concerning the reversibility of potential effects of certain EDCs on male mate calling behavior, as well as the assessment whether modifications of male mating calls affect the attractiveness of males for females should reveal the biological relevance of exposure of *X. laevis* to particular, environmentally relevant EDCs.

2 Methods

All adult male and female *X. laevis* used in the present study were obtained from the breeding stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB, Berlin, Germany). Frogs were kept in mixed-sex groups of up to 25 frogs in 80 L aquaria at 20 °C ± 1 °C. The light/dark cycle was 12:12 h and frogs were fed a commercial fish diet (Fisch-Fit, Interquell, Wehringen, Germany) twice a week. The German State Office of Health and Social Affairs (LaGeSo, Berlin, Germany) reviewed and approved all procedures for this study.

2.1 Exposure of male *Xenopus laevis* to endocrine disrupting compounds with (anti)androgenic and (anti)estrogenic modes of action

2.1.1 Subjects

To examine the effects of EDCs with (anti)androgenic and (anti)estrogenic MOAs on male mate calling behavior of *X. laevis*, 10 male *X. laevis* (age: 2 – 5 years; weight: 17.1 g ± 5.4 g; snout-to-vent length: 6.3 cm ± 0.7 cm) were used per treatment group, adding up to 240 adult males in total. During experiments, frogs were fed twice a week (Fisch-Fit, Interquell, Wehringen, Germany) and water temperature was monitored daily.

2.1.2 Chemicals and exposure concentrations

MDHT (> 98 %), VIN (> 99 %), EE2 (≥ 98 %), TAM (≥ 99 %) and ICI (> 98 %), were purchased from Sigma Aldrich (Steinheim, Germany). To prepare stock solutions, test substances were dissolved in dimethyl sulfoxide (DMSO, ≥ 99.5 %; Carl Roth GmbH, Karlsruhe, Germany). Each exposure substance, except ICI, was tested at three different concentrations: 10^{-6} M or 10^{-7} M, as well as 10^{-8} M and 10^{-10} M (Tab. 1). EE2 was additionally tested in a second test series, determining its effects at the concentrations 10^{-10} M, 10^{-11} M and 10^{-12} M (Tab. 1). TAM was tested individually at the above mentioned concentrations, as well as in a simultaneous treatment with EE2 (Tab. 1). ICI, on the other hand, was only tested in a simultaneous treatment with EE2 (Tab. 1). DMSO concentration in each test tanks was 0.001 %. Control animals were exposed to DMSO only (0.001 %). To save control animals, two exposure substances (MDHT + TAM, EE2 + VIN, as well as the simultaneous

exposure of EE2 + TAM and EE2 + ICI) were tested in parallel using one set of controls.

Tab. 1: Exposure concentrations and modes of action of the different exposure substances.

Exposure substance (molecular weight)	**Mode of action**	**Injection with human chorionic gonadotropin**	**Exposure concentration** [M]	**Exposure concentration** [µg/L or ng/L]
MDHT (304.5 g/mol)	androgenic	-	10^{-10} M	30.45 ng/L
			10^{-8} M	3.045 µg/L
			10^{-7} M	30.45 µg/L
VIN (286.1 g/mol)	antiandrogenic	✓	10^{-10} M	28.61 ng/L
			10^{-8} M	2.861 µg/L
			10^{-6} M	286.1 µg/L
EE2 (296.4 g/mol)	Estrogenic	✓	10^{-10} M	29.64 ng/L
			10^{-8} M	2.964 µg/L
			10^{-6} M	296.4 µg/L
			10^{-12} M	0.296 ng/L
			10^{-11} M	2.964 ng/L
			10^{-10} M	29.64 ng/L
TAM (371.5 g/mol)	antiestrogenic	-	10^{-10} M	37.15 ng/L
			10^{-8} M	3.715 µg/L
			10^{-7} M	37.15 µg/L
TAM + EE2	estrogenic and antiestrogenic	✓	EE2: 10^{-10} M	EE2: 29.64 ng/L
			TAM: 10^{-7} M	TAM: 37.15 µg/L
ICI + EE2 (ICI: 606.8 g/mol)	estrogenic and antiestrogenic	✓	EE2: 10^{-10} M	EE2: 29.64 ng/L
			ICI: 10^{-7} M	ICI: 60.68 µg/L

2.1.3 Exposure and treatment

For exposure, individual male frogs were transferred into 60 L glass tanks (50 cm × 40 cm × 30 cm), where they remained visually and acoustically isolated from each other. Crosstalk between animals was prevented by affixing pyramid profiled acoustic foam around each tank. Test tanks, containing 20 L of distilled water supplemented with 5 g marine salt (Tropic Marin Meersalz, Tagis, Dreieich, Germany), were randomly assigned to the respective exposure treatments (Tab. 1; Fig. 14). Within the test tanks, frogs were allowed to acclimatize for 72 h.

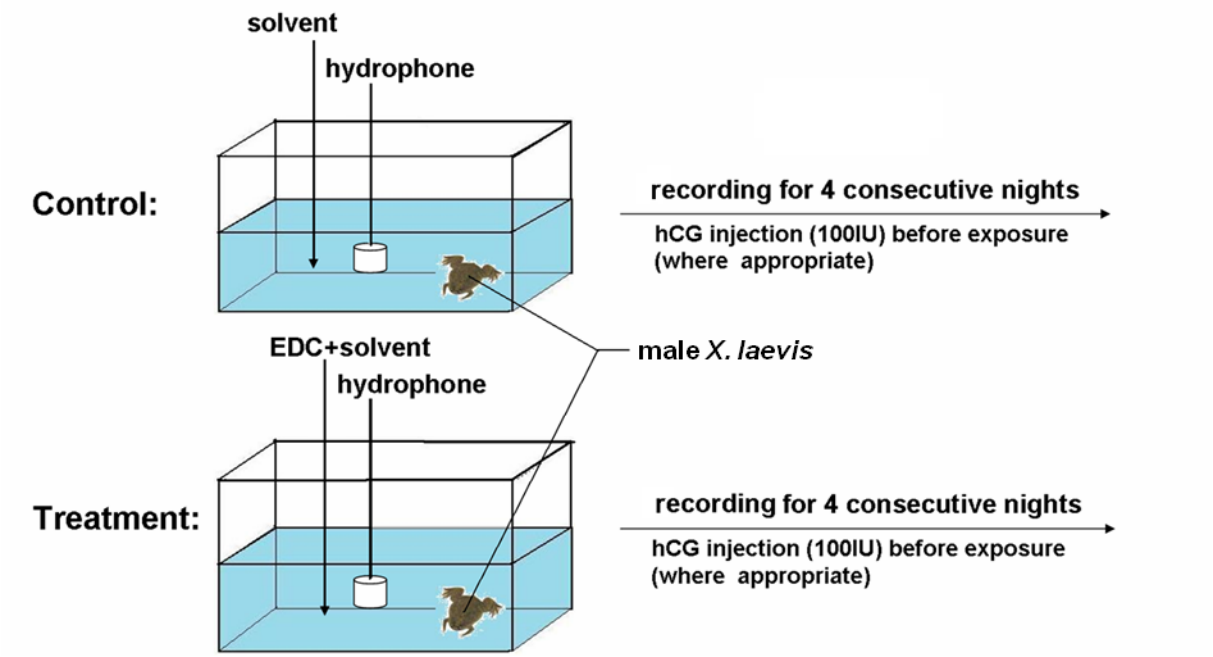

Fig. 14: Experimental setup.

Because VIN and EE2 are assumed to reduce male sexual activity (Colbert et al. 2005; Saaristo et al. 2009, 2010a), animals assigned to these treatments as well as the appertaining control frogs, were injected with 100 IU human chorionic gonadotropin (hCG; dissolved in 50 µl distilled water, injected to the dorsal lymph sac; Tab. 1) to stimulate a basic mate calling behavior (Kelley and Pfaff 1976; Wetzel and Kelley 1983). TAM and MDHT treated frogs and their appertaining control animals did not receive any hCG injections, because the stimulatory effect of hCG might mask the potential stimulating effects of MDHT and TAM (Wetzel and Kelley 1983; Urbatzka et al. 2006b, 2007). Animals simultaneously exposed to EE2 and TAM or ICI received hCG injections (dissolved in 50 µl distilled water, injected to the dorsal lymph sac; Tab. 1).

Subsequently, frogs were exposed to the respective EDC at the adequate concentration or volumetric equivalent dose of solvent only (0.001% DMSO) within their test tanks. Exposures lasted for 96 h. EDC and solvent control solutions were prepared every other day when rearing water and chemicals were renewed. At the end of the experiment, animals were anesthetized in 0.01% tricaine methanesulfonate (MS222; Sigma Aldrich, Steinheim, Germany) for four minutes. They were weighted, and the snout-to-vent length was measured. Afterwards, frogs were euthanized by decapitation. Blood, testes, brain and liver samples were taken for further biochemical and molecular biological analyses (see 2.3).

2.2 Mate calling behavior of male *Xenopus laevis*

2.2.1 Acoustic monitoring

From the day of exposure until the end of the experiment, the acoustic behavior of the frogs was recorded at night-time (18:00 – 06:00 h). For this purpose, a hydrophone (Sensor Technology SQ26, Nauta, Milano, Italy) was placed in each tank. Hydrophones were used in combination with an external multichannel interface (Tascam US-1641, TEAC Corporation, Tokyo, Japan) that was connected to a desktop computer. An automated, trigger-controlled recording in a frequency range of 0.5 – 3.5 kHz was performed for each tank by using the Avisoft Recorder software (Avisoft, Berlin, Germany). Channel-specific recordings were stored as separate wave files.

2.2.2 Call analyses

For quantitative analyses, the absolute calling activity and relative proportions of each of the different call types were calculated for every frog on each of the four recorded nights. This calculation was done by visually inspecting individual spectrograms, identifying different call types and measuring their duration using Avisoft SasLab software (Avisoft, Berlin, Germany). The absolute duration of a call type uttered was then divided by the total vocalization time of the individual frog.

Subsequently, advertisement calls were analyzed in more detail. Because clicks of the fast trill parts of ACs overlapped due to the rapidity of the trills (Fig. 5 and 15), analyses of these call parts were not possible. Nevertheless, characteristic temporal features of the slow trills, such as mean duration of individual clicks, duration between individual clicks within one trill (ICD), mean click rate, mean number of clicks and duration of an entire call, were measured (Avisoft SasLab). Furthermore, spectral features of the entire ACs, including mean peak frequency, mean bandwidth, mean number of accentuated clicks (Fig. 15), and the mean entropy of the calls were examined. Analyses were performed for each of the four recorded nights and the number of examined clicks did not exceed 10,000 clicks per night. Group medians and interquartile ranges (IQR) were calculated. To avoid potential observer bias, all analyses were conducted in a blind manner regarding exposure treatment.

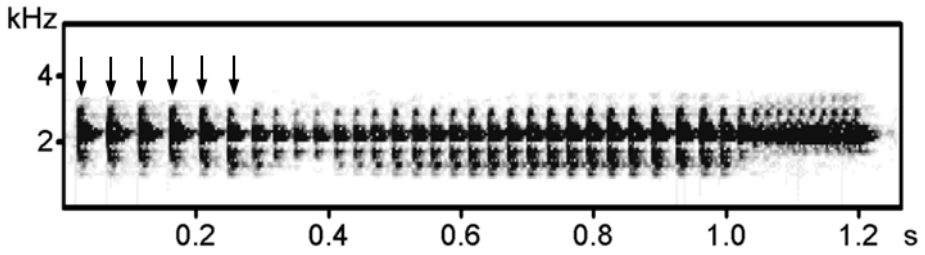

Fig. 15: Spectrogram of an advertisement call of a male *Xenopus laevis*. Vertical arrows indicate accentuated clicks (clicks with pronounced sound intensity).

2.2.3 Statistical analysis

Data did not fit the assumptions for normality or homogeneity of variances. Nonetheless, the use of general linear mixed models (GLMMs) was suitable because residuals from models as response variables showed normal distributions. GLMMs are an extension of general linear models that allow both random and fixed effects as well as covariates to be fitted to the model. GLMMs account for unequal sample sizes and for repeated measurement of the same individual by including subject as a random factor in the model (Pinheiro and Bates 2000). GLMMs were used to analyze the overall treatment effect of the different EDCs on the various measured parameters of the male mate calling behavior of *X. laevis.* Subjects were set as random factor and the covariates body weight, body length and water temperature were also included in the model. Parameters showing a significant variation between treatment concentrations were further analyzed using post-hoc pairwise comparisons (GLMMs) to determine where the variation existed. False discovery rate (FDR) was applied to control for type I errors from conducting multiple tests Nicheols and Hayasaka 2003). All statistical analyses were performed using PASW Statistics 17 (SPSS Inc., Chicago, Illinois, USA).

2.3 Biomolecular and biochemical biomarkers for the detection of (anti)androgenic and (anti)estrogenic endocrine disrupting compounds

To compare sensitivity of the behavioral endpoints measured with standardized biomolecular and biochemical biomarkers, plasma hormone concentrations (T, E2 and P4) as well as various gene expression analyses were performed.

2.3.1 Plasma hormone concentrations

2.3.1.1 Sampling

After exposure, male *X. laevis* were sacrificed and blood was sampled in EDTA-containing 1.5 mL SafeLock tubes (Eppendorf, Hamburg). Blood samples were centrifuged at 12.000 *g* for 4 min. Plasma was transferred into new 1.5 mL SafeLock tubes and samples were frozen immediately in liquid nitrogen. Samples were stored at -80 °C until further processing.

2.3.1.2 Sex steroid determination

Plasma hormone concentrations of E2, T and P4 were determined using enzyme immunoassays (EIAs; Cayman Chemicals, Ann Arbour, MI, USA). EIAs are based on competitive binding of hormone within the sample and an added hormone-acetyl cholinesterase conjugate (tracer) to a limited number of steroid-specific antiserum binding sites. The amount of tracer is always held constant, while the amount of hormone in the samples varies. Hence, the amount of tracer bound to the antiserum is inversely proportional to the concentration of hormone in the well (Estradiol EIA kit – handbook). The detection limits of these assays are 19 pg/mL for E2, 6 pg/mL for T, and 10 pg/mL for P (Cayman Chemicals, Ann Arbour, MI, USA).
Frozen plasma samples (100 µl per sample) were melted on ice and transferred to 5ml glass vials (Carl Roth GmbH, Karlsruhe, Germany). To extract hormones, 1 mL diethyl ether (> 99.5 %, Carl Roth GmbH, Karlsruhe, Germany) was added to each vial and the solution was mixed for 15 s. By freezing the aqueous phase for 30 – 45 at -80 °C min, the organic phase including plasma hormones could be transferred to new vials. This procedure was repeated twice and the respective organic fractions were pooled. Ether was allowed to evaporate within 24 h at room temperature and residuals were reconstituted in EIA buffer (Tab. 2 and 3).

Tab. 2: Sex steroid determination - list of reagents to be supplied by the user.

Reagent	Description	Supplier
diethyl ether	>99.5% p.a.	Carl Roth GmbH, Karlsruhe, Germany
Ultrapure water	deionised water, free of organic contaminants	Cayman Chemicals, Ann Arbour, MI, USA

EIAs for E2, T, and P4 were performed in parallel using the same reconstituted sample. To match the concentration ranges of the EIA, samples were diluted with EIA buffer according to previous studies applying respective assays in *X. laevis* (Urbatzka et al. 2007). EIAs were performed as recommended by the manufacturer (Cayman Chemicals, Ann Arbor, MI, USA; protocol see Annex 1).

Tab. 3: Preparation of working solutions for use in EIAs.

Solution	Preparation
EIA buffer (1x)	Dilute buffer concentrate (10x) with ultrapure water (1:10); make sure to rinse the vial to remove any precipitated salts; EIA buffer (1x) can be stored at 4 °C.
Washing buffer (1x)	Dilute buffer concentrate (400x) with ultrapure water (1:400) and add Polysorbate 20 (0.5 ml/L); washing buffer (1x) can be stored at 4 °C.
Antiserum	Reconstitute respective antiserum with EIA buffer (100 dtn EIA Antiserum per 6 mL EIA buffer).
Tracer	Reconstitute tracer with EIA buffer (100 dtn EIA tracer per 6 mL EIA buffer).
Hormone standards	Dilute EIA hormone standards with ultrapure water (1:10) to obtain the bulk standard (40 ng/mL); prepare standard series by serial dilution of bulk standard with EIA buffer; use within 24 h.
Ellman's solution	Reconstitute Ellman's solution with EIA buffer immediately before use (100 dtn Ellman's reagent per 20 mL Ultrapure water).

Samples were assayed in duplicates and absorbance was measured at 412 nm with a 96-well plate reader (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland). Resulting data included total enzyme activity, background absorbance caused by Ellman's solution, non-immunological binding of the tracer to the well, as well as the maximum amount of tracer that the antibody can bind. Hormone concentration of plasma samples was calculated with respect to the dilution of the sample using the standard curve.

2.3.1.3 Statistical analysis

Statistical differences between treatments were determined using One-way ANOVA followed by Dunnett's post-hoc test. Normality of data was ensured by Kolmogorov-Smirnoff-test. Not normally distributed data was tested with non-parametric tests (Kruskal-Wallis test and *post hoc* Mann-Whitney-U test).

2.3.2 Gene expression analysis

Genes are expressed, when DNA is transcribed into mRNA, which in turn is translated into the gene product (protein) (Fig. 16).

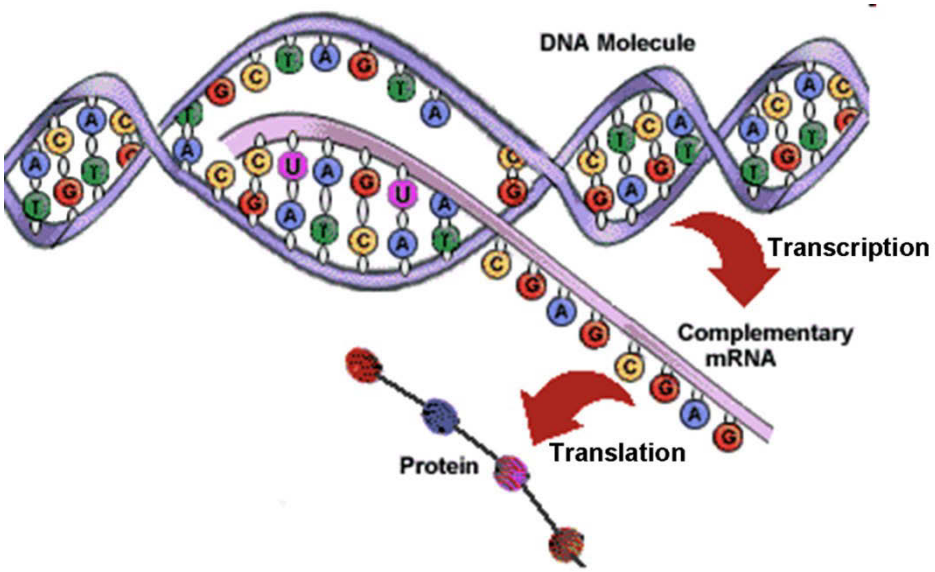

Fig. 16: Schematic of the transcription (DNA to mRNA) and translation (mRNA to protein) process (modified from http://schoolworkhelper.net/2010/07/protein-synthesis-transcription/).

The concentration of a gene-specific mRNA is proportional to the amount of gene product, and hence to the activity of this gene within a sample. Using reverse transcription polymerase chain reaction (RT-PCR), mRNA is reverse transcribed into robust complementaryDNA (cDNA). Using gene-specific primers, defined cDNA sequences are then amplified using a PCR technique called real-time PCR. In real-time PCRs, as in general PCRs, defined sequences of a piece of DNA (or cDNA) are amplified exponentially, resulting in millions of copies of this sequence. Real-time PCRs, however, additionally quantify the amount of DNA copies to predict the activity of the examined gene.

2.3.2.1 Sampling

After exposure, male *X. laevis* were sacrificed and brain, liver and gonad samples were taken and transferred into 1.5 mL SafeLock tubes (Eppendorf, Hamburg). Samples were immediately frozen in liquid nitrogen and stored at -80°C for further processing.

2.3.2.2 Extraction of total RNA

Isolation of total-RNA from individual brain, gonad and liver samples was done using QIAzol Lysis Reagent (Qiagen, Hilden, Germany; Tab 4), a mixture of phenol and guanidine thiocyanate that lyses homogenized tissues without destroying the RNA. Total RNA isolation was performed as recommended by the manufacturer (Quiagen, Hilden, Germany; protocol see Annex 2).

Tab. 4: List of reagents for isolation of total-RNA.

Reagent	Description	Supplier
QIAzol Lysis Reagent	Monophasic solution of phenol and guanidine thiocyanate, lysis of fatty tissues and inhibition of RNases.	Qiagen, Hilden, Germany
Chloroform	≥ 99 %	Carl Roth GmbH, Karlsruhe, Germany
Isopropanol	2-Propanol, ≥ 99.5 %	Carl Roth GmbH, Karlsruhe, Germany
Ethanol	70 %	Carl Roth GmbH, Karlsruhe, Germany
RNase free water	Distilled water, treated with 0.01% diethyl-pyrocarbonate (DEPC) for 12 h at room temperature, sterilised by autoclaving and stored at 4°C.	DEPC (≥ 97 %) from Carl Roth GmbH, Karlsruhe, Germany

2.3.2.3 Determination of total RNA concentration and purity

RNA concentrations and purity were determined spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Thermo Fisher Scientific, Wilmington, USA). The sample RNA concentration is given in ng/µL based on absorbance at 260 nm. The purity of the sample is given by the ratio of sample absorbance at 260 nm and 280 nm. A ratio around 2.0 is generally accepted as “pure” for RNA. A considerably lower ratio might indicate the presence of contaminants that absorb strongly at or near 280 nm, like some proteins or phenol. RNA purity scores of all samples were 1.93 ± 0.09. Thus, the quality of RNA in all analyzed samples was good.

2.3.2.4 Determination of RNA integrity

The quality of total-RNA samples was further ensured by identifying degradation of total RNA within a subset of samples from each extraction using RNA 6000 Nano LabChips (Agilent Technologies, Waldbronn, Germany) analyzed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany; Tab. 5). For determination of RNA concentration, 1 µL of sample (RNA concentration between 25 – 500 ng/µL) was used. Chips were prepared according to the manufacturer's instructions (Agilent Bioanalyzer RNA 6000 Nano Assay Manual; protocol see Annex 3).

The 2100 expert software (version B.02.05.SI360; Agilent Technologies, Waldbronn, Germany) calculates RNA integrity numbers (RIN) and assigns one RIN score (1 – 10) per sample. A RIN score of 10 indicates a perfectly intact RNA, while a score of 1 represents highly degraded RNA (Schroeder et al. 2006). The RIN scores of all samples analyzed in this study were ≥ 9.0. Thus, the quality of RNA in all analyzed samples was good.

Tab. 5: List of reagents for analyzing total-RNA quality using 6000 Nano LabChips.

Reagent	Description	Supplier
RNase free water	RNase-free water prepared without the use of DEPC	Qiagen, Hilden, Germany
RNA 6000 ladder	Set of six RNA transcripts with lengths of 0.2, 0.5, 1.0, 2.0, 4.0, and 6.0 kb.	Agilent Technologies, Waldbronn, Germany
RNaseZAP	For electrode decontamination	Agilent Technologies, Waldbronn, Germany
RNA 6000 Nano gel matrix	RNA gel matrix	Agilent Technologies, Waldbronn, Germany
RNA 6000 Nano dye concentrate	RNA dye	Agilent Technologies, Waldbronn, Germany
RNA 6000 Nano Marker	RNA marker	Agilent Technologies, Waldbronn, Germany

2.3.2.5 DNase treatment of total RNA from liver samples

To remove potential genomic DNA contamination of liver samples, samples were digested using DNase I (amplification grade, Invitrogen, Karlsruhe, Germany). DNase

I digests single- and double-stranded DNA to oligodesoxyribonuleotides. The DNase treatment protocol can be found in Annex 4.

2.3.2.6 cDNA synthesis by reverse transcription

Reactions were carried out using a thermal cycler (Biometra, Göttingen, Germany) and all reagents and mixtures (Tab. 6) were prepared on ice. RT-PCRs were conducted according to the following protocol.

Tab. 6: List of reagents for RT-PCR.

Reagent	Description	Supplier
RNase free water	Distilled water, treated with 0.01% diethylpyrocarbonate (DEPC; ≥ 97 %) for 12 h at room temperature, sterilised by autoclaving and stored at 4°C.	DEPC from Carl Roth GmbH, Karlsruhe, Germany
RNase free water	RNase-free water prepared without the use of DEPC	Qiagen, Hilden, Germany
Oligo(dT)12-18 Primer	2.5 µM; suitable for use in first-strand cDNA synthesis with reverse transcriptase. The primer hybridizes to the poly(A) tail of mRNA.	Invitrogen, Karlsruhe, Germany
dNTP-solution	10 mM each dNTP; ideal for reverse transcription (first-strand cDNA synthesis) and PCR.	Invitrogen, Karlsruhe, Germany
AMV-RT-buffer (10x)	750 mM Tris-HCl (pH 8.3), 200 mM $(NH_4)_2SO_4$, 25 mM $MgCl_2$, 1% Tween 20.	Invitrogen, Karlsruhe, Germany
AMV-RT	20 units / µL; AMV-RT is commonly used to synthesize a DNA copy of input RNA (cDNA synthesis) and is utilized in conjunction with Taq DNA Polymerase for RT-PCR.	Invitrogen, Karlsruhe, Germany

8 µL of sample solution (=1µg RNA) and 9 µL RNase free water, as well as 3 µL ploy-dt-primer were heated at 70 °C for 3 min to let the primers anneal to the poly-A sequence of the mRNA. For annealing of poly-dt-primers to DNase I digested RNA samples, 11 µL sample solution (~ 1 µg RNA), 6 µL RNase free water, as well as 3 µL poly-dt-primer were heated for 3 min at 70 °C. Samples were then cooled on ice. Then, 5 µL RNase free water, 3 µL AMV-RT-buffer (10x), 1.5 µL dNTPs and 0.5 µL AMV-RT were added to each sample and samples were incubated at 37 °C for 60 min, heated up to 94 °C for 2 min and cooled down to 10°C. Samples were stored at -20°C until further processing.

2.3.2.7 Gene expression analysis using real-time PCR

Real-time PCRs were conducted using the Mx3005p qPCR cycler (Stratagene, Amsterdam, Netherlands). To quantify DNA, the cycler measures fluorescence of SYBR® Green I (Peqlab, Erlangen, Germany) during each PCR cycle. SYBR® Green I binds to double-stranded DNA, thus its fluorescence amplifies proportional to the amplification of the double-stranded DNA. To amplify only defined sequences of interest (LH and FSH in brain samples, aromatase, 5α-reductase 1 and 5α-reductase 2 in gonad samples and Vtg in liver samples) specific primers (250 nM) were used (Urbatzka et al. 2010; Lorenz et al. 2011b; Lorenz and Hermelink personal communication; Tab. 7). Specificity of PCR products was analyzed by Urbatzka and colleagues (2010), who ran the bands on an agarose gel, extracted and sequenced them and subsequently compared sequences by BLAST and confirmed the specificity of amplified PCR products.

Tab. 7: Primer used for gene expression analysis using real-time PCR.

Target	**Primer sequence, forward (5'–3')**	**Primer sequence, reverse (5'–3')**
Luteinizing hormone (LH), β subunit – brain	ACACTgACgCTTCTggggTTCTAC	gATTgggCAgTCgTCTTTCTCT
Follicle stimulating hormone (FSH), β subunit – brain	TgCTCgTTCTgTgTTggAAgATg	CCTgTTTgATgAgTggATgCTTTg
Aromatase (ARO) – gonads	CggTTCCATATCgTTACTTCC	gCATCTTCCTCTCAATgTCTg
Steroid 5α reductase 1 (Red 1), Polypeptide 1 – gonads	CTgAACCTCTTggCTATg	gATgCCTAACTCggATTg
Steroid 5α reductase 2 (Red 2), Polypeptide 2 – gonads	CTTATCCTgCTgCTTATg	AgTCCTgTggAAATAgTg
Vitellogenin (Vtg) – liver	AAgACCAAgAgCCCAgAAg	ggAgAgCATCAAgCAAAC
Elongation factor 1 α (EF) - housekeeping gene	TgCCATTgTTgACATgATCCC	TACTATTAAACTCTgATggCC

Real-time-PCRs were conducted according to the following protocol using 2x SensiMix™ SYBR Low-ROX Kit (Peqlab, Erlangen, Germany):

In 0.2 mL Real-time-PCR tubes (Peqlab, Erlangen, Germany), 2 µL of 10x diluted cDNA solution (template) were mixed with 6.25 µL 2x SensiMix™ SYBR Low-ROX and 0.125 µL of the respective forward and reverse primer, respectively. All samples were assayed in duplicates. Besides the cDNA samples, a calibrator sample (triplicate), a H_2O negative control and two minus RT-PCR samples (RT-PCR sample without addition of AMV-RT) were included in each real-time-PCR assessment. Tubes were put into the cycler and amplification was performed (Tab. 8). Assay specificity was ensured performing a melting curve analysis after each run (Tab. 8).

Tab. 8: Thermal profile of cDNA amplification by real-time PCR.

Cycles	Temperature [°C]	Time	Notes
1	95	10 min	DNA denaturing, Polymerase activation
40	95	17 s	DNA denaturing
	62	25 s	Primer annealing
	72	25 s	Elongation
1	95	40 s	Melt-profile analysis
	55	30 s	
	95	0 s	
1	25	30 s → pause	Pause

Amplification efficiency was tested by preparing 5-fold serial dilution series of pooled cDNA from all samples of one RNA extraction and running these samples in a real-time PCR. Cycle thresholds (C_T) were plotted against the logarithm of the cDNA dilution (Pfaffl 2001) and regression lines were calculated. Slopes from regression lines (regression fit equations) were used to determine amplification efficiency for each pooled sample:

$$\text{Efficiency} = 10^{(-1/\text{slope})}$$

All amplification efficiencies ranged between 1.89 and 2.07.

MxPro software (Stratagene, Waldbronn, Germany) was used to analyse real-time PCR data using the 'comparative calibrator' setting of the software. According to Pfaffl (2001), the $\Delta\Delta C_T$ method was applied with respect to the calibrator samples

and corrected for PCR efficiencies of the target gene. C_T values were normalized by subtracting the respective C_T value of the housekeeping gene (EF).

2.3.2.8 Statistical analysis

Data were tested for normal distribution, using Kruskal-Wallis tests. Since all data were normally distributed, one-way ANOVA followed by *post hoc* Dunnett's T3 tests were performed to identify significant differences between treatments. Homogenity of variances was ensured using Levene's test.

2.4 Reversibility of altered spectral and temporal parameters of male advertisement calls due to exposure to 17α-ethinylestradiol

2.4.1 Subjects

Ten adult male *X. laevis*, 3 years of age (weight: 15.9 g ± 2.2 g; snout-to-vent length: 6.3 cm ± 0.7 cm), were used for determining the reversibility of altered spectral and temporal parameters of male advertisement calls due to EE2 exposure. During experiments, frogs were fed twice a week (Fisch-Fit, Interquell, Wehringen, Germany) and water temperature was monitored daily.

2.4.2 Exposure treatment, acoustic monitoring and call analyses

Reversibility of altered call features was tested by recording the nocturnal calling behavior of male *X. laevis* at night time (18:00 – 06:00 h) for four consecutive nights. *X. laevis* were individually placed into 10 L glass tanks and stimulated by 100 IU hCG (dissolved in 50 µL dH_2O, injected in the dorsal lymph sac) (i) two weeks before, as well as (ii) during a four-night EE2 exposure at 2.96 µg/L (details on exposure treatment and call monitoring: see 2.1.2 – 2.1.4). After EE2 exposure, frogs were kept in distilled water (10 L) supplemented with 2.5 g of marine salt (Tropic Marin Meersalz, Tagis, Dreieich, Germany) without being exposed to any EDC. (iii) Four and (iv) six weeks after EE2 exposure, the nocturnal calling behavior of the frogs, stimulated with 100 IU hCG, was recorded and analyzed again.

2.4.3 Statistical analysis

Because data were not normally distributed, statistical overall differences between the various time points were determined using nonparametric Friedman tests. To analyze statistical differences between single time points, Wilcoxon-signed rank tests were used as *post hoc* tests.

2.5 Biological relevance of alterations of spectral and temporal parameters of male advertisement calls due to 17α-ethinylestradiol exposure

In *X. laevis* and other frogs, it was suggested that sexual identity recognition of male and female frogs, as well as mate identification is based on temporal calling parameters (Loftus-Hill and Littlejohn 1971; Gerhardt 1978; Picker 1983; Bush et al. 2002; Schul and Bush 2002; Vignal and Kelley 2007) and that spectral cues convey information about the attractiveness of a call (Vignal and Kelley 2007). Hence, alterations of temporal or spectral parameters due to EE2 contamination might influence the attractiveness of exposed males towards females and as a result might decrease the reproductive success of males and females. To address this issue, female choice experiments were performed using a y-maze apparatus (Fig. 17).

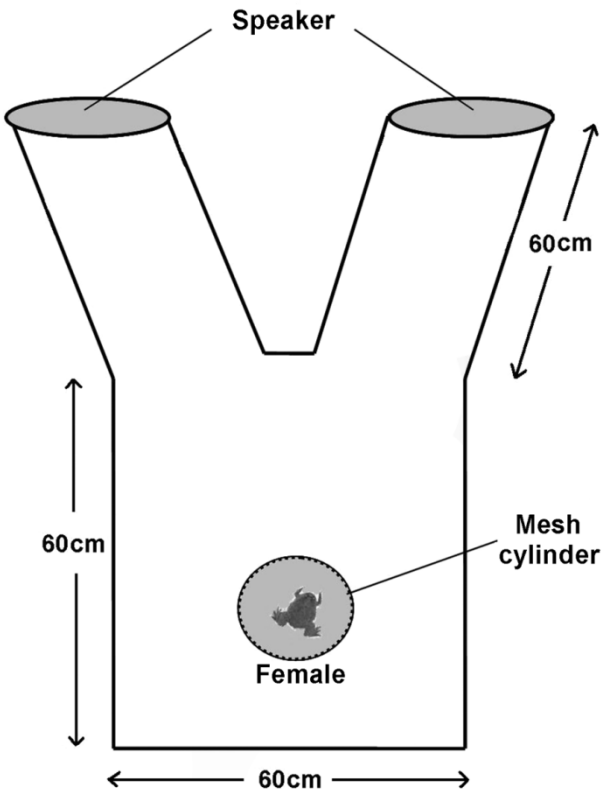

Fig. 17: Scheme of the y-maze apparatus (water depth 25 cm).

2.5.1 Subjects

To examine female responses to male AC, 30 female *X. laevis* (5 years of age) were tested. Females were kept in 10 L glass tanks for 12 h to acclimatize. 3 h before the first test series, females were stimulated with 600 IU hCG (dissolved in 100 µL dH_2O). The second test series started approximately 1 h after the first series. After performing choice tests, females were put back in their tank and left overnight until oviposition. Females were reintroduced to the breeding stock three weeks later.

2.5.2 Playback presentation

To determine whether receptive females can discriminate calls from noise and whether they prefer male calls over noise, females (N= 10) were given the choice between AC playback versus white noise in the first experiment. In the second female choice experiment, females (N= 10) chose between two simultaneous AC playbacks from a male once being exposed to EE2 and once being held under control conditions. In the third playback experiment, females (N= 10) were presented with only one playback at a time: once an AC playback of an unexposed control male and in another test trial a playback of AC uttered by the same male while being exposed to EE2. To control for potential confounding side preferences, playback order was counterbalanced between trials.

2.5.3 Playbacks

All playbacks were derived from recordings of male AC produced in response to hCG stimulation. For ‘noise’ playbacks, white noise files without any vocalizations were used. Playbacks lasted 16 min and consisted of sixteen 1 min repetitions of AC bouts. Playbacks were standardized concerning the number of calls to avoid preferences on the basis of performance-related traits (Gil and Gahr 2002). As average ± SD playbacks had 847.6 ± 35.3 advertisement calls with a frequency of 2111.2 ± 114.0 kHz, bandwidth of 1748.3 ± 434.5 kHz and an amplitude of -31.5 ± 5.9 dB. None of these parameters differed significantly between the two treatments compared (Wilcoxon signed-ranks tests: N = 20).

2.5.4 Female choice test

Female choice tests took place in an insulated climate chamber in the dark. Experiments were digitally videotaped (OSCAR CCD Camera, 640 x 480 Pixel) with

the help of an infrared headlight, and analyzed in a 'blind fashion' regarding female identity and trial number.

To start a test trial, a female was placed in a mesh cylinder at the end of the y-maze (120 cm x 100 cm and 20 cm high, water depth = 25 cm; Fig. 17). At the other end there were two speakers (UW30, frequency range 100 – 10,000 Hz; impedance 8 Ω), one in each arm of the y-maze. Within the mesh cylinder, females were able to hear playbacks from both arms. After an acclimation period of 90 s, playback(s) started. After another 30 s, the cylinder was lifted from outside the chamber and for 15 min, females could move freely within the y-maze. For analyzing females' relative attraction females' retention times in a 10 cm distance to a speaker and the time females moved when being close to a speaker (10 cm) were measured.

2.5.5 Statistical analysis

To determine significant differences in active retention times in close proximity to the speakers, overall Friedman tests and *post hoc* paired Wilcoxon signed-ranks tests were applied.

3 Results

Besides the four call types AC, chirping, growling and ticking, a fifth call type was determined during this study, which was termed rasping and which had not been analyzed previously. Rasping is a long trill (> 5 s) consisting of up to several hundreds of clicks with a click duration between 5 ms and 20 ms, an ICD between 15 ms and 100 ms and a frequency range between 1.8 kHz and 2.3 kHz (Fig. 18).

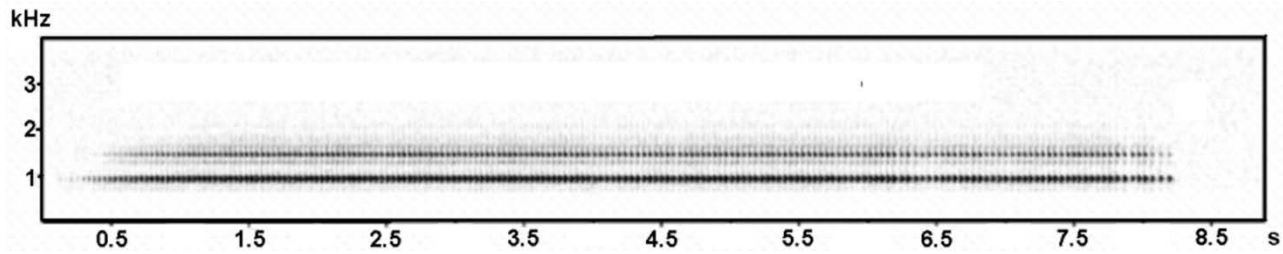

Fig. 18: Spectrogram of the call type rasping.

HCG-stimulated males predominantly produced ACs and chirping, which are call types that indicate a sexually aroused state of the male. Isolated, sham injected males, on the other hand, predominantly produced growling, ticking and rasping calls, call types that are not indicating sexual arousal. Answer and amplectant calls were neither produced by hCG- nor sham-injected isolated males in the laboratory.

3.1 Effects of exposure to (anti)androgenic and (anti)estrogenic endocrine disrupting compounds on the mate calling behavior of male *Xenopus laevis*

While animals were exposed to the respective EDCs, various parameters of the calling behavior of male *X. laevis* were analyzed to assess whether this behavior might be suitable as biomarker for the detection of EDCs with (anti)androgenic and (anti)estrogenic MOAs.

3.1.1 Androgenic 17α-methyldihydrotestosterone

MDHT at all concentrations significantly increased the percentages of ACs indicating a sexually aroused state of the male ($p < 0.05$, Fig. 19 a), although the total vocal output of the males did not differ between treatments (Tab. 9). The call type chirping was not produced by any frog.

Tab. 9: Effects of exposure to different concentrations of 17α-methyldihydrotestosterone (MDHT) on male calling behavior of *Xenopus laevis.* Values are medians (IQR). Treatments did not differ significantly from controls.

Treatment	Night (after exposure)	Total vocal output (min)	Growling (%)	Ticking (%)
Solvent control	1	133.2 (1.9 – 225.1)	0.1 (0.4 – 1.6)	0.1 (0.1 – 0.2)
	2	112.2 (0.3 – 154.6)	0.4 (0.3 – 1.0)	0 (0 – 0.1)
	3	84.6 (0.8 – 129.1)	0.3 (0.2 – 1.5)	0 (0 – 0.1)
	4	26.8 (0.1 – 82.1)	0.7 (0.3 – 0.9)	0 (0 – 0.1)
MDHT 30.45 ng/L	1	40.8 (16.2 – 197.4)	0.1 (0.1 – 0.4)	0 (0 – 0)
	2	52.4 (28.5 – 228.1)	0.1 (0 – 0.5)	0 (0 – 0)
	3	87.6 (17.4 – 118.7)	0 (0 – 0.2)	0 (0 – 0)
	4	22.9 (8.4 – 70.1)	0.3 (0 – 1.1)	0 (0 – 0)
MDHT 3.05 µg/L	1	115.2 (10.9 – 277.7)	0.2 (0 – 0.5)	0 (0 – 0.1)
	2	172.7 (27.4 – 297.8)	0.2 (0 – 0.6)	0 (0 – 0.3)
	3	147.9 (33.8 – 226.4)	0.3 (0 – 0.5)	0 (0 – 0.2)
	4	76.2 (10.9 – 100.3)	0 (0 – 0.3)	0 (0 –0)
MDHT 30.45 µg/L	1	62.1 (3.2 – 129.0)	0.3 (0 – 0.5)	0 (0 – 0.1)
	2	39.4 (0.7 – 95.6)	0 (0 – 0.3)	0 (0 – 0.1)
	3	43.3 (7.3 – 109.8)	0.6 (0.3 – 0.8)	0 (0 – 0)
	4	46.8 (7.6 – 113.9)	0.4 (0.2 – 0.5)	0 (0 – 0)

The percentages of rasping, a call type not indicating sexual arousal, on the other hand, significantly decreased in all MDHT treated groups ($p < 0.05$, Fig. 19 b). The proportions of growling and male ticking were not affected by MDHT (Tab. 9). Body weight, body length and water temperature did not influence the portions of the different call types. Temporal and spectral analyses of ACs did not reveal any significant effects of MDHT exposure on the measured parameters.

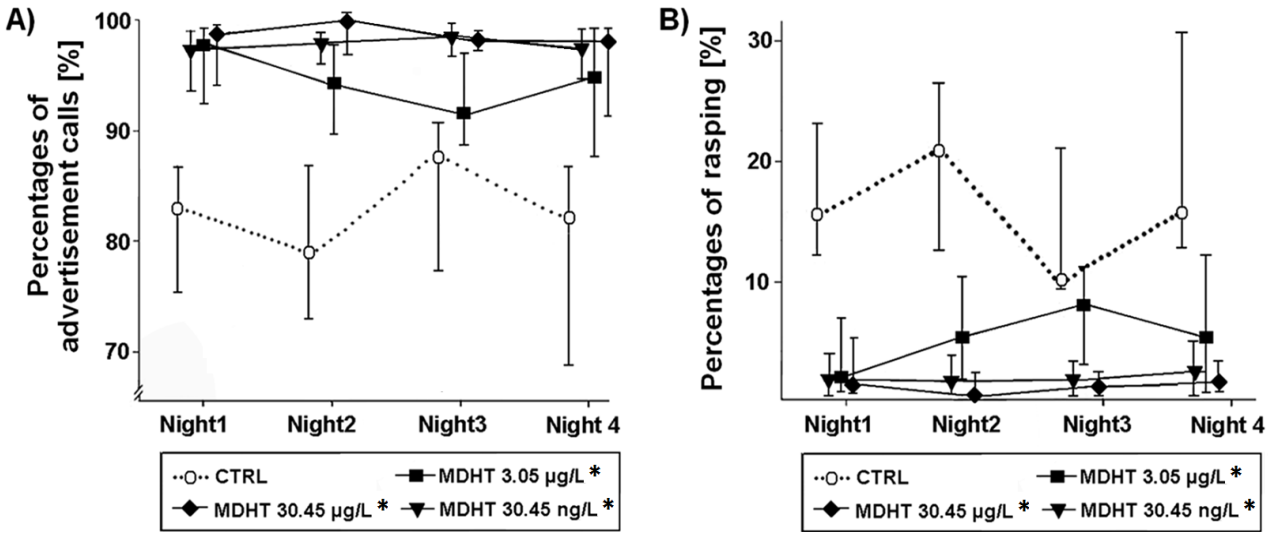

Fig. 19: Percentages (median (IQR) of (A) advertisement calls and (B) the call type rasping produced by male *Xenopus laevis* exposed to 17α-methyldihydrotestosterone (MDHT). Proportions are given for each treatment in each night (n= 10 per treatment). Statistical differences were determined using General Linear Mixed models. Significant differences from solvent control (CTRL) are marked by asterisks (* $p \leq 0.05$).

Already during the first night, MDHT exposure at all concentrations tested led to significantly enhanced percentages of advertisement calls ($p < 0.05$; Fig. 19 a) and significantly lower percentages of rasping ($p < 0.05$; Fig. 19 b).

3.1.2 Antiandrogenic vinclozolin

Body weight, body length and water temperature did not have any significant effect on the production rates of the different call types. Frogs that were sham-injected with distilled water called significantly less than hCG-injected counterparts and frogs treated with VIN at 28.6 µg/L and 28.6 ng/L ($p \leq 0.01$; Fig. 20). Males exposed to VIN at the highest concentration (286.1 µg/L), by contrast, vocalized significantly less than hCG-injected controls ($p \leq 0.01$; Tab. 10; Fig. 20).

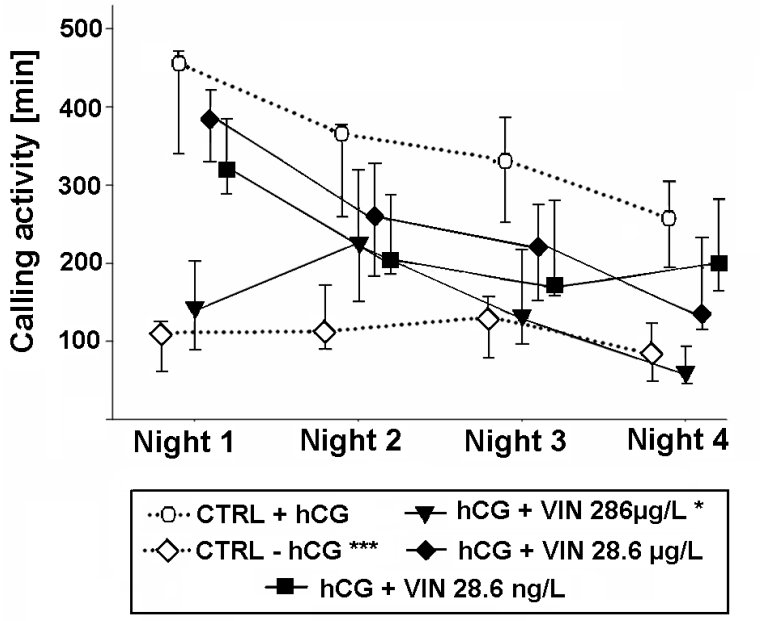

Fig. 20: Calling activity per night and per frog (median (IQR)) shown for all vinclozolin (VIN) treatment groups (n = 10). HCG was administered in the morning before the first recording session. Statistical differences were determined using General Linear Mixed models. Significant differences from solvent control with hCG injection (CTRL + hCG) are marked by asterisks (* $p \leq 0.05$; *** $p \leq 0.001$).

VIN exposure at 28.6 ng/L and 2.86 µg/L led to a significant decrease in percentages of calls that were ACs ($p \leq 0.01$; Tab. 10; Fig. 21). Likewise, animals of the VIN (286.1 µg/L)-treated group uttered lower relative percentages of calls that were ACs, but changes were not significant (Tab. 10; Fig. 21). Sham-injected frogs uttered also lower percentages of ACs ($p \leq 0.01$; Tab. 10; Fig. 21). Regarding the first night of exposure, all MDHT treatments resulted in lower portions of AC ($p \leq 0.05$; Fig. 21). The call type chirping was not produced by any treatment group.

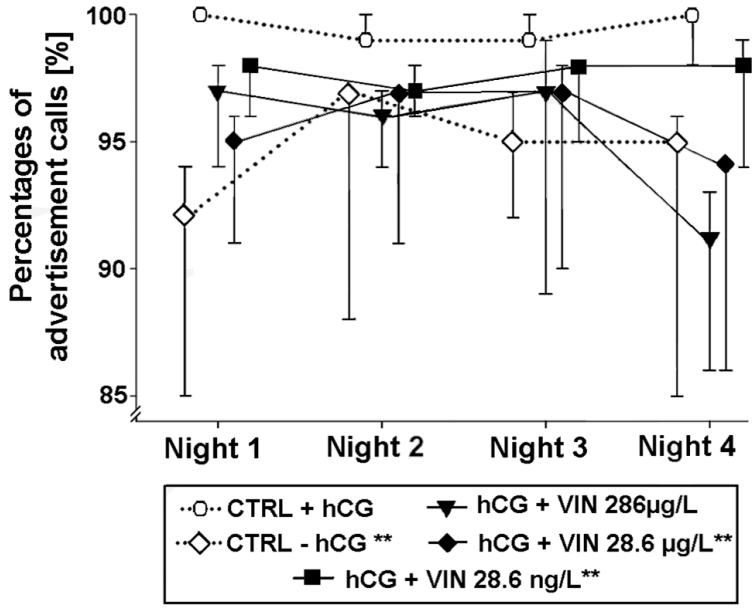

Fig. 21: Percentages (median (IQR)) of advertisement calls produced by male *Xenopus laevis* exposed to vinclozolin (VIN). Percentages are given for each treatment in each night (n= 10 per treatment). Statistical differences were determined using General Linear Mixed models. Significant differences from solvent control with hCG injection (CTRL + hCG) are marked by asterisks (** $p \leq 0.01$).

Percentages of the call type growling were significantly higher in all VIN treated groups ($p \leq 0.01$; Tab. 10). Furthermore, VIN at 28.6 µg/L and 28.6 ng/L led to increased portions of ticking during every recorded night, but the overall treatment comparison was not significant (Tab. 10).

Regarding the percentages of growling during the first night of exposure, all VIN treatment groups showed significantly higher portions than controls ($p \leq 0.01$; Tab. 10). Rasping was not affected by any treatment (Tab. 10).

Tab. 10: Effects of exposure to different concentrations of vinclozolin (VIN) on male calling behavior of *Xenopus laevis.* HCG and sham injections were given in the morning before the first recording session. Values are median (IQR). Treatments did not differ significantly from controls.

Treatment	Night (after exposure)	Advertisement call (%)	Growling (%)	Ticking (%)	Rasping (%)
Solvent control - hCG	1	92.1 (71.7 – 94.2)	1.3 (0.7 – 2.2)	0 (0 – 0)	2.5 (1.9 – 5.0)
	2	97.1 (88.6 – 97.7)	0 (0 – 0)	0 (0 – 0)	3.4 (2.0 – 12.3)
	3	95.6 (92 – 97.6)	0 (0 – 1.0)	0 (0 – 0.1)	2.9 (1.9 – 6.9)
	4	95.7 (85.8 – 96.9)	0 (0 – 0.8)	0 (0 – 0)	0 (0 – 5.1)
Solvent control + hCG	1	100 (99.2 – 100)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
	2	99.5 (99.3 – 100)	0 (0 – 0)	0 (0 – 0)	0 (0 – 1.0)
	3	99.2 (98.6 – 99.9)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0.9)
	4	99.1 (98.5 – 99.8)	0 (0 – 0)	0 (0 – 0)	0 (0 – 2.1)
VIN 286.1 µg/L	1	97.4 (89.1 – 97.7)	1.0 (1.0 – 2.1)	0 (0 – 0.1)	0.5 (0 – 5.4)
	2	97.0 (93.8 – 97.9)	1.1 (0.9 – 2.2)	0 (0 – 1.4)	1.3 (0.9 – 3.1)
	3	98.5 (89.9 – 99.4)	0.9 (0 – 1.0)	0 (0 – 1.9)	0 (0 – 2.5)
	4	91.3 (86.0 – 93.6)	4.5 (2.1 – 9.3)	0 (0 – 3.0)	1.5 (0.7 – 5.0)
VIN 2.86 µg/L	1	95.7 (91.3 – 96.8)	2.7 (1.3 – 3.3)	0.5 (0.3 – 2.4)	1.1 (0.8 – 5.0)
	2	97.3 (91.0 – 97.9)	2.1 (2.0 – 6.3)	0.1 (0 – 1.3)	1.1 (1.0 – 3.3)
	3	98.1 (89.8 – 99.7)	1.7 (1.1 – 7.1)	0.5 (0.2 – 1.7)	1.5 (1.0 – 3.9)
	4	94.2 (86.8 – 94.9)	2.4 (2.0 – 8.1)	1.0 (0.9 – 3.4)	1.4 (1.0 – 4.6)
VIN 28.6 ng/L	1	98.4 (96.2 – 98.9)	1 (0.9 – 2.4)	0 (0 – 1.1)	1.3 (0.9 – 2.1)
	2	96.8 (96.1 – 98.6)	0 (0 – 1.0)	1.3 (1.3 – 1.9)	1.5 (1.0 – 2.3)
	3	97.8 (90.0 – 97.4)	1.2 (1.1 – 1.9)	1.5 (1.0 – 1.8)	1.6 (0 – 3.0)
	4	98.2 (94.1 – 99.3)	1.1 (0.8 – 4.0)	0.9 (0.6 – 4.0)	0 (0 – 4.1)

Most spectral parameters were not influenced by any treatment. The mean peak frequency and the mean bandwidth, as well as the entropy of the calls were not affected. Nevertheless, animals of all VIN treated groups produced significantly fewer accentuated clicks than hCG-injected control frogs ($P \leq 0.01$; Fig. 22 a). Sham injected animals, however, did not differ from hCG-injected controls (Fig. 22 a). Body weight, body length and water temperature did not affect the temporal and spectral parameters.

Similarly, most of the temporal parameters of the male mate calling behavior of *X. laevis* were not affected by any of the VIN treatments. Nevertheless, the mean duration of clicks in the slow trills of ACs were decreased by VIN treatment at all three concentrations (Fig. 22 b). Temporal parameters of sham injected animals did not differ from hCG-injected controls (Fig. 22 b).

While the number of accentuated clicks was not significantly affected during the first night of exposure, the click duration of AC was a significantly decreased by VIN exposure at all concentrations ($P \leq 0.05$; Tab. 10).

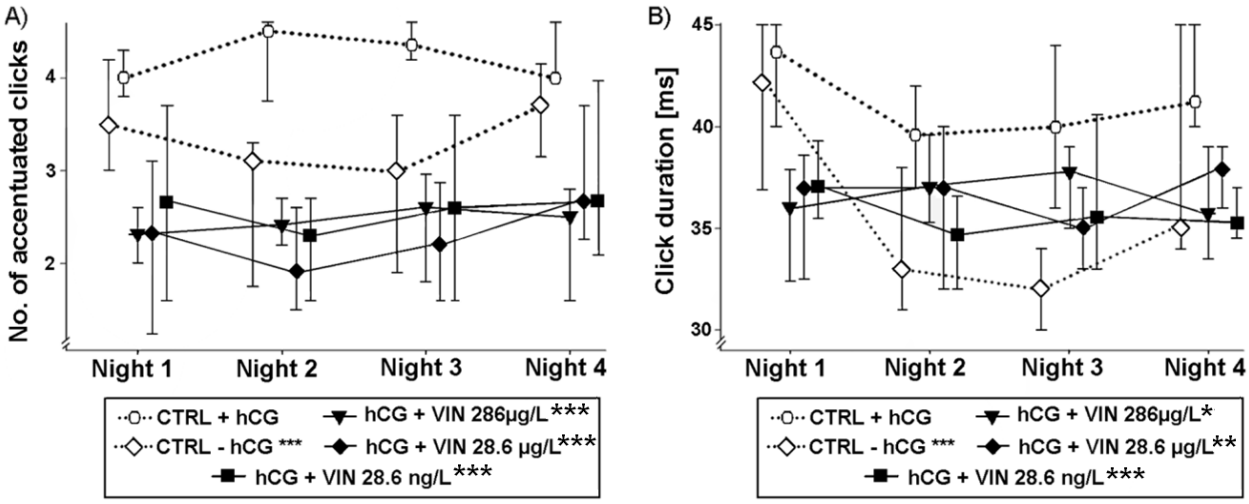

Fig. 22: (A) Number of accentuated clicks (median (IQR)) and (B) click duration (median (IQR)) for each vinclozolin (VIN) treatment in each night. HCG was administered in the morning before the first recording session. Statistical differences were determined using General Linear Mixed models. Significant differences from solvent control with hCG injection (CTRL + hCG) are marked by asterisks (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

3.1.3 Estrogenic 17α-ethinylestradiol

The total vocal output of the males was not affected by EE2 exposure (Tab. 11). Nevertheless, the absolute calling activity of frogs that received a sham injection was significantly lower than the total vocal output of hCG-injected animals ($p \leq 0.001$; Tab. 11). Interestingly, in comparison to unexposed frogs, EE2 exposed individuals generally uttered significantly lower percentages of advertisement calls ($p \leq 0.05$; Fig. 23) and significantly higher proportions of rasping calls ($p \leq 0.05$), similarly as in sham-injected animals ($p \leq 0.01$; Fig. 24). EE2 exposure at 2.96 µg/L, 29.6 ng/L, 2.96 ng/L and 0.296 ng/L caused lower percentages of ACs already during the first night of exposure ($p \leq 0.01$), whereas rasping was only affected by EE2 at 2.96 µg/L and 29.6 ng/L during the first exposure night ($p \leq 0.05$; Fig. 23 and 24).

The other call types (chirping, growling and ticking) were not affected by EE2 exposure or hCG injection (Tab. 11).

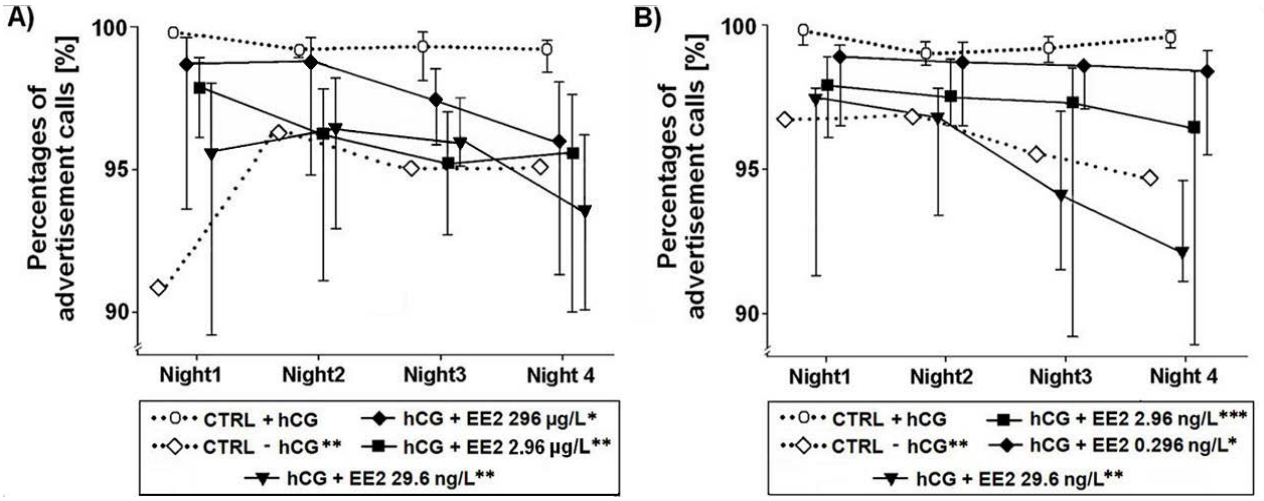

Fig. 23: Percentages of advertisement calls produced by male *Xenopus laevis* exposed to 17α-ethinylestradiol (EE2). Median (IQR) (n=10 per treatment) for EE2 exposure concentrations of (A) 296 µg/L, 2.96 µg/L and 29.6 ng/L and (B) 29.6 ng/L, 2.96 ng/L and 0.296 ng/L. Statistical differences were determined using General Linear Mixed models. Significant differences from solvent control (CTRL) are marked by asterisks (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

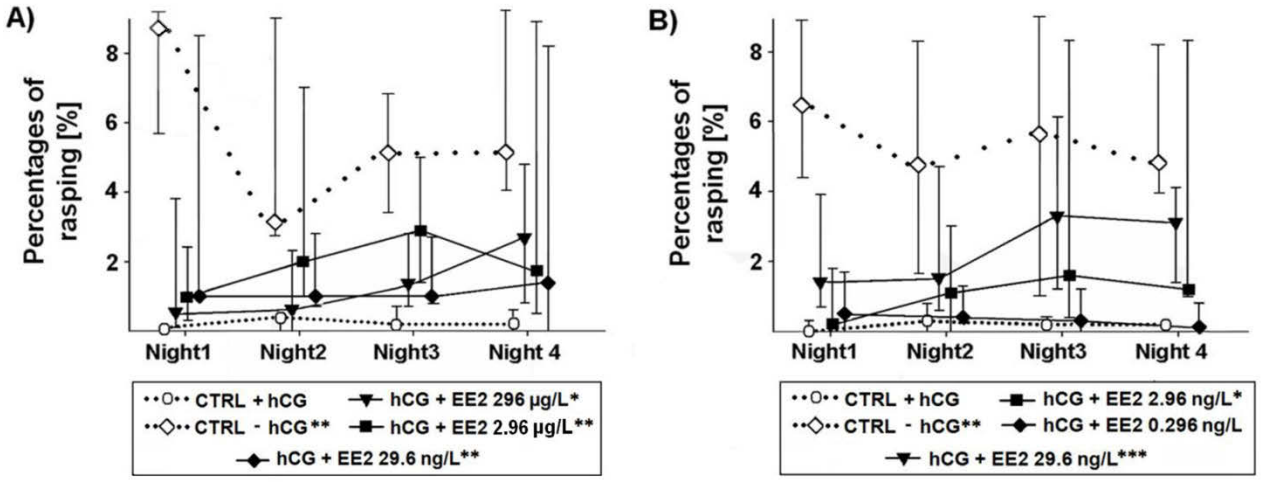

Fig. 24: Percentages of the call type rasping produced by male *Xenopus laevis* exposed to 17α-ethinylestradiol (EE2). Median (IQR) (n= 10 per treatment) for EE2 exposure concentrations of (A) 296 µg/L, 2.96 µg/L and 29.6 ng/L and (B) 29.6 ng/L, 2.96 ng/L and 0.296 ng/L. Statistical differences were determined using General Linear Mixed models. Significant differences from solvent control (CTRL) + human chorionic gonadotropin (hCG) treatment are marked by asterisks (* $p \leq 0.05$; *** $p \leq 0.001$).

EE2 exposure at any of the five different concentrations resulted in significantly lower numbers of accentuated clicks at the beginning of the AC ($p \leq 0.01$; Fig. 25 and 26). The number of accentuated clicks produced by sham-injected frogs, however, did not differ from hCG-injected animals (Fig. 26). Lower numbers of accentuated clicks due to EE2 exposure at 2.96 µg/L, 29.6 ng/L, 2.96 ng/L and 0.296 ng/L were detected already during the first night of exposure ($p \leq 0.05$; Fig. 26).

Tab. 11: Effects of exposure to different concentrations of 17α-ethinylestradiol (EE2) on male calling behavior of *Xenopus laevis*. hCG injections were given in the morning before the first recording session. Values are median (IQR). Treatments did not differ significantly from controls.

Treatment		Night (after exposure)	Total vocal output (min)	Chirping (%)	Growling (%)	Ticking (%)
First trial	Solvent control - hCG	1	79.0 (11.3 – 142.1)	0 (0 – 0)	1.3 (0.7 – 2.2)	0 (0 – 0)
		2	107.4 (35.7 – 152.3)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
		3	93.6 (16.7 – 182.7)	0 (0 – 0)	0 (0 – 1.0)	0 (0 – 0.1)
		4	65.5 (1.7 – 96.2)	0 (0 – 0)	0 (0 – 0.8)	0 (0 – 0)
	Solvent control + hCG	1	456.0 (403.1 – 497.2)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
		2	365.8 (243.7 – 414.6)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
		3	331.8 (202.8 – 411.4)	0.1 (0 – 0.1)	0 (0 – 0)	0 (0 – 0)
		4	258.1 (136.8 – 344.6)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
	EE2 29.64 ng/L	1	189.0 (70.5 – 357.5)	0 (0 – 0)	0.4 (0 – 0.7)	0 (0 – 0)
		2	270.1 (124.4 – 402.6)	0 (0 – 0)	0.4 (0 – 2.0)	0 (0 – 0)
		3	215.8 (79.1 – 339.0)	0 (0 – 0)	0.7 (0.1 – 1.7)	0 (0 – 0)
		4	179.7 (90.4 – 220.8)	0 (0 – 0)	1.1 (0.1 – 2.8)	0 (0 – 0)
	EE2 2.96 µg/L	1	318.9 (211.8 – 443.3)	0 (0 – 0)	0.4 (0 – 0.6)	0 (0 – 0.5)
		2	159.7 (105.9 – 278.6)	0 (0 – 0)	0.01 (0 – 0.013)	0 (0 – 0.8)
		3	102.8 (41.8 – 183.6)	0 (0 – 0)	1.0 (0.3 – 1.9)	0.9 (0.4 – 1.3)
		4	91.7 (33.1 – 203.7)	0 (0 – 0)	1.3 (0.5 – 1.9)	0.8 (0 –1.4)
	EE2 296.4 µg/L	1	240.3 (10.3 – 481.2)	0 (0 – 0)	0.2 (0 – 0.6)	0.3 (0 – 1.6)
		2	208.2 (40.0 – 354.4)	0 (0 – 0)	0.2 (0 – 0.8)	1.3 (0 – 2.2)
		3	160.0 (26.7 – 278.8)	0 (0 – 0)	0.5 (0.2 – 0.8)	1.6 (0.2 – 2.7)
		4	37.7 (0.7 – 126.9)	0 (0 – 0)	0.1 (0 – 1.2)	3.7 (0 – 7.3)
Second trial	Solvent control - hCG	1	30.9 (0.05 – 113.1)	0 (0 – 0)	0.9 (0.3 – 1.3)	0.2 (0.1 – 0.4)
		2	27.1 (0.1 – 131.6)	0 (0 – 0)	0.5 (0.3 – 0.9)	0.1 (0 – 0.1)
		3	1.1 (0.7 – 18.3)	0 (0 – 0)	0.1 (0 – 1.3)	0.2 (0 – 0.8)
		4	73.9 (0.2 – 151.6)	0 (0 – 0)	0.8 (0.2 – 2.0)	0.2 (0.1 – 0.3)
	Solvent control + hCG	1	94.1 (6.0 – 179.3)	0 (0 – 0)	0 (0 – 0.1)	0 (0 – 0)
		2	169.2 (41.4 – 225.1)	0 (0 – 0.1)	0.1 (0 – 0.3)	0 (0 – 0)
		3	149.4 (74.5 – 237.5)	0 (0 – 0)	0 (0 – 0.6)	0 (0 – 0)
		4	118.2 (39.5 – 176.7)	0 (0 – 0)	0.1 (0 – 0.2)	0 (0 – 0)
	EE2 29.64 ng/L	1	82.5 (39.2 – 135.5)	0 (0 – 0)	1.0 (0.8 – 3.0)	0.2 (0.1 – 0.4)
		2	91.4 (47.5 – 166.5)	0 (0 – 0)	0.8 (0.5 – 1.5)	0.4 (0 – 0.6)
		3	129.8 (95.7 – 181.6)	0 (0 – 0)	0.9 (0.5 – 2.1)	0.3 (0.1 – 0.8)
		4	124.4 (50.1 –162.5)	0 (0 – 0)	1.2 (0.8 – 4.4)	0.3 (0 – 0.7)
	EE2 2.96 ng/L	1	29.0 (3.4 – 231.5)	0 (0 – 0)	0.4 (0 – 1.7)	0.1 (0 – 0.4)
		2	85.5 (8.9 – 265.1)	0 (0 – 0)	0.7 (0 – 1.4)	0.1 (0 – 0.3)
		3	125.3 (63.3 – 263.0)	0 (0 – 0)	0.5 (0.2 – 1.2)	0.1 (0 – 0.2)
		4	155.4 (32.0 – 235.0)	0 (0 – 0)	0.9 (0.2 – 4.5)	0.1 (0 –0.1)
	EE2 0.296 ng/L	1	129.4 (27.6 – 198.0)	0 (0 – 0)	0.5 (0.3 – 1.8)	0 (0 – 0.2)
		2	136.1 (26.4 – 166.5)	0 (0 – 0)	0.4 (0.2 – 1.5)	0 (0 – 0.1)
		3	139.5 (29.3 – 187.2)	0 (0 – 0)	1.1 (0.8 – 1.2)	0 (0 – 0.1)
		4	50.9 (28.2 – 142.1)	0 (0 – 0)	0.9 (0.7 – 1.4)	0.1 (0 – 0.2)

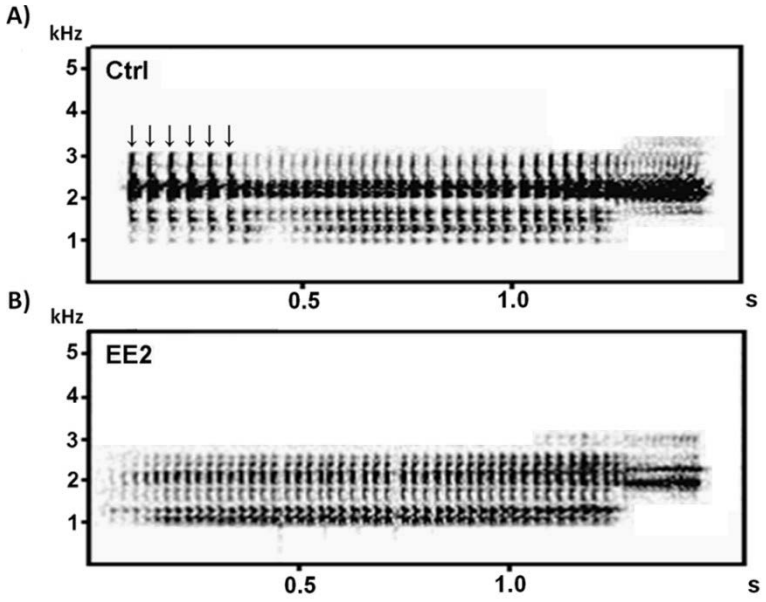

Fig. 25: Spectrogram of an advertisement call of (A) an unexposed control male (CTRL) with six accentuated clicks at the beginning of the call (indicated by vertical arrows) and (B) an EE2 exposed male (2.96 µg/L) with no accentuated clicks.

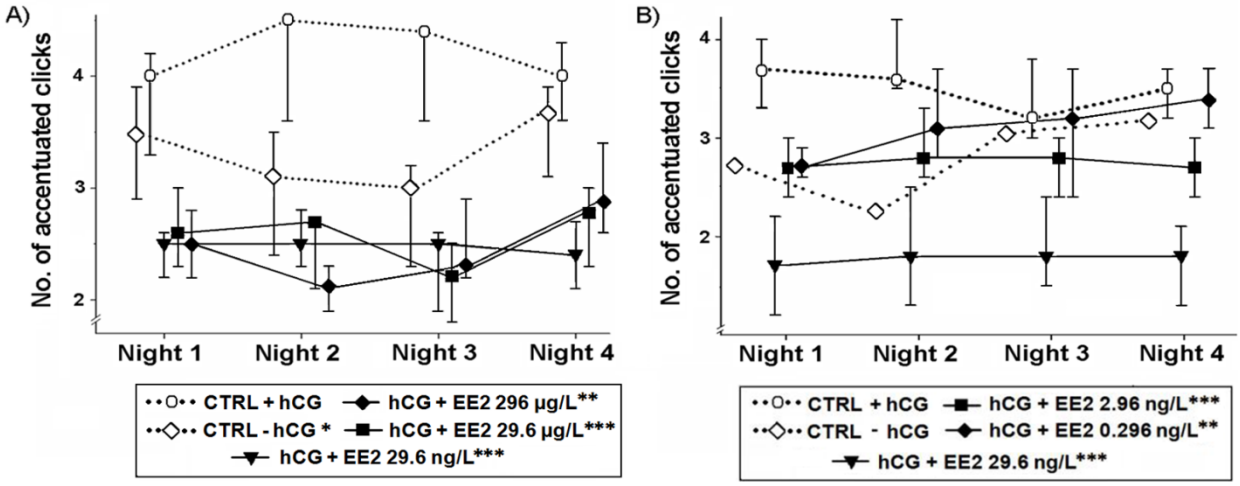

Fig. 26: No. of accentuated clicks within male advertisement calls produced by male *Xenopus laevis* exposed to 17α-ethinylestradiol (EE2). Median (IQR) (n=10 per treatment) for EE2 exposure concentrations of (A) 296 µg/L, 2.96 µg/L and 29.6 ng/L and (B) 29.6 ng/L, 2.96 ng/L and 0.296 ng/L. Statistical differences were determined using General Linear Mixed models. Significant differences from solvent control (CTRL) + human chorionic gonadotropin (hCG) treatment are marked by asterisks (** $p \leq 0.01$; *** $p \leq 0.001$).

Further analyses revealed that the duration of clicks of AC was significantly reduced in all except the lowest treatment concentration (0.296 ng/L EE2) ($p \leq 0.05$; Fig. 27). This effect already occurred during the first night of exposure ($p \leq 0.05$; Fig. 27). The click duration of sham-injected frogs was lower compared to hCG-injected animals, however, differences were not significant. Body weight and body length, as well as water temperature did not affect any of these parameters.

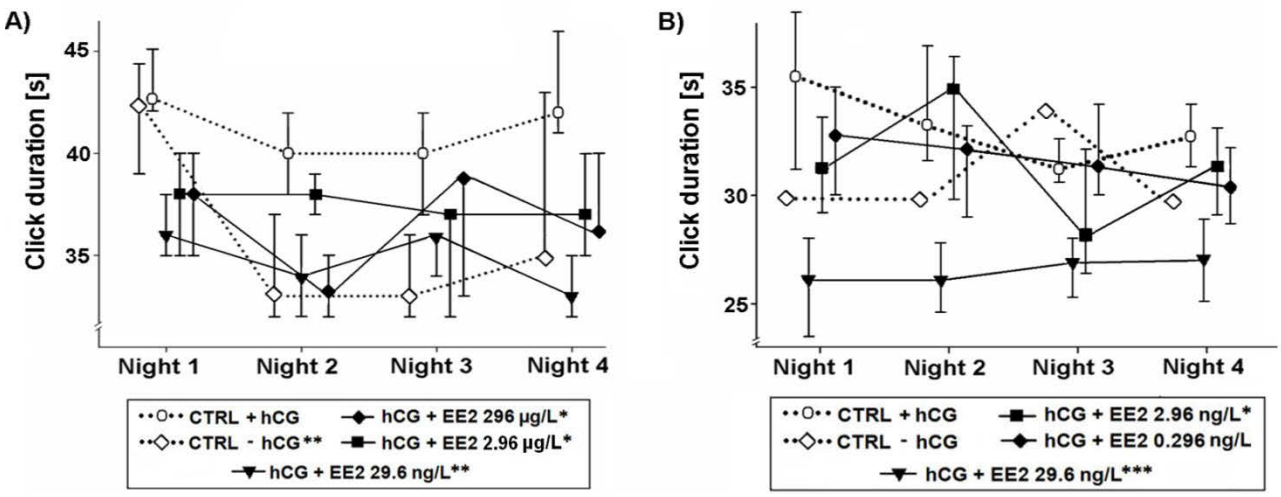

Fig. 27: Duration of clicks of male advertisement calls produced by male *Xenopus laevis* exposed to 17α-ethinylestradiol (EE2). Median (IQR) (n=10 per treatment) for EE2 exposure concentrations of (A) 296 µg/L, 2.96 µg/L and 29.6 ng/L and (B) 29.6 ng/L, 2.96 ng/L and 0.296 ng/L. Statistical differences were determined using General Linear Mixed models. Significant differences from solvent control (CTRL) + human chorionic gonadotropin (hCG) treatment are marked by asterisks (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

3.1.4 Antiestrogenic tamoxifen and fulvestrant

Sole TAM exposure at any tested concentration neither affected the total vocal output of the frogs, nor the composition of different call types (see Annex 5, Tab. A1), nor any spectral and temporal parameters of the advertisement call. Sole EE2 exposure (29.6 ng/L), on the other hand, resulted in a lower percentage of advertisement calls ($p \leq 0.01$; Fig. 28 a), a higher percentage of the call type rasping ($p \leq 0.05$; Fig. 28 b), a shorter duration of clicks within ACs ($p \leq 0.01$; Fig. 28 d), and a lower number of accentuated clicks ($p \leq 0.001$; Fig. 28 c), as it was shown in 3.1.3.

If frogs were simultaneously exposed to EE2 at 29.6 ng/L and a 1000-fold TAM concentration (37.1 µg/L), some of the EE2 effects vanished, such as the lower portions of advertisement calls ($p \geq 0.05$; Fig. 28 a), the higher percentages of rasping ($p \geq 0.05$; Fig. 28 b) and the shorter click durations ($p \geq 0.05$; Fig. 28 c).

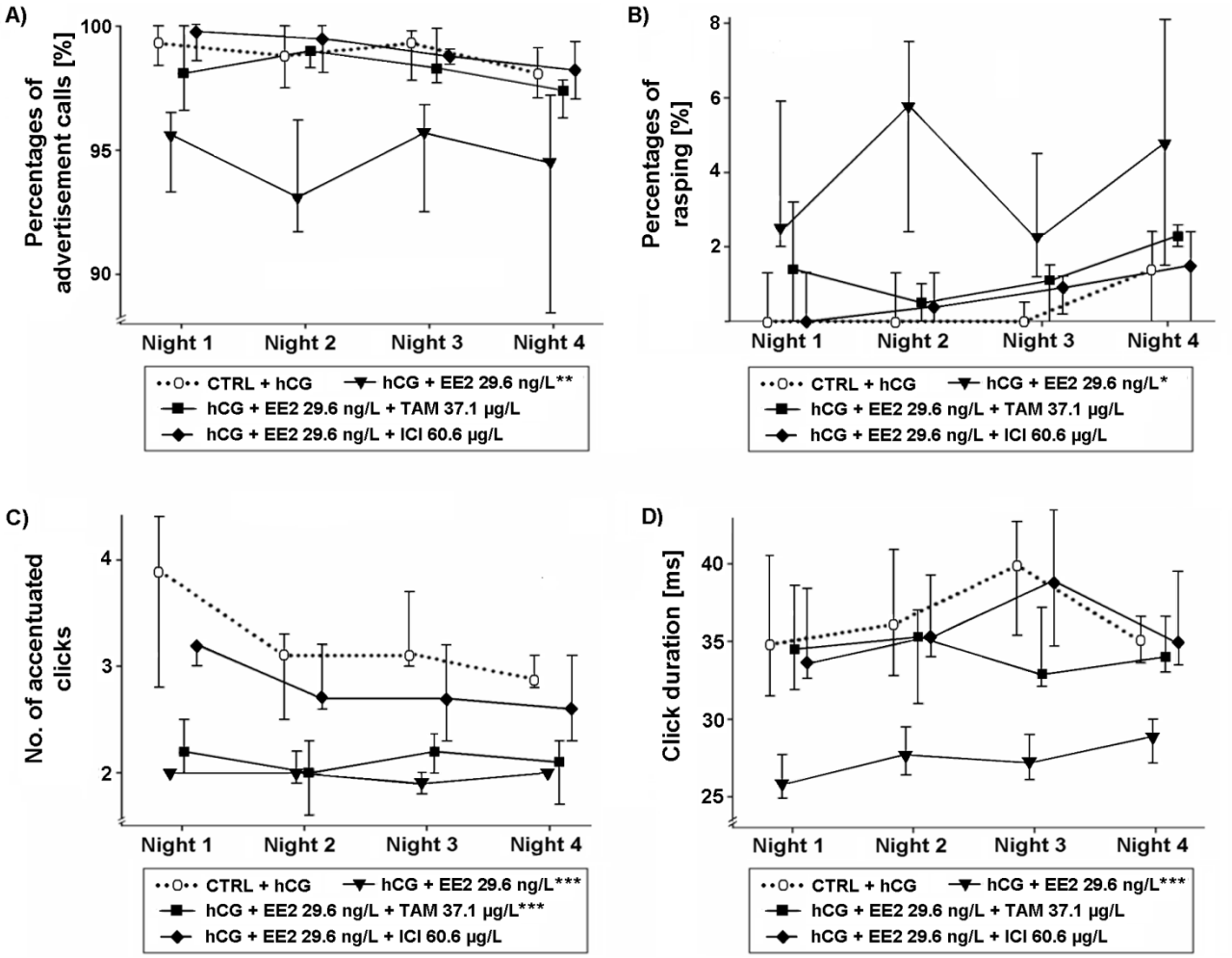

Fig. 28: Median (IQR) (n=10 per treatment) of (A) percentages of advertisement calls, (B) percentages of the call type rasping, (C) number of accentuated clicks and (D) duration of clicks of male advertisement calls produced by male *Xenopus laevis* exposed to 17α-ethinylestradiol (EE2) or a mixture of EE2 and tamoxifen (EE2+TAM) or EE2 and fulvestrant (EE2+ICI). Statistical differences were determined using General Linear Mixed models. Significant differences from solvent control (CTRL) + human chorionic gonadotropin (hCG) treatment are marked by asterisks (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

Simultaneous exposure to EE2 (29.6 ng/L) and a 1000-fold ICI (pure estrogen antagonist) concentration (60.7 µg/L), on the other hand, erased all measured EE2 effects: the lower portions of advertisement calls ($p \geq 0.05$; Fig. 28 a), the higher percentages of the call type rasping ($p \geq 0.05$; Fig. 28 b), the lower number of accentuated clicks ($p \geq 0.05$; Fig. 28 c), as well as the lower click durations ($p \geq 0.05$; Fig. 28 d).

EE2 effects occurred already during the first night of exposure ($p \leq 0.05$; Fig. 28, see also 3.1.3). TAM co-exposure cancelled out the lower proportions of advertisement calls and the higher percentages of the call type rasping during this night ($p \geq 0.05$; Fig. 28), whereas ICI co-exposure erased all EE2 effects during the first night of exposure ($p \geq 0.05$; Fig. 28).

3.2 Effects of exposure to (anti)androgenic and (anti)estrogenic endocrine disrupting compounds on biomolecular and biochemical biomarkers

The most relevant biomolecular and biochemical biomarker for EDCs being analyzed during this study were plasma hormone concentrations of T, E2 and P4, as well as the mRNA expression of aromatase (ARO) and steroid reductase type 1 (RED1) and type 2 (RED2) in gonad samples, LH and FSH in brain samples and Vtg in liver samples.

3.2.1 Plasma sex steroid levels

Plasma levels of T, E2 and P4, respectively, were not significantly affected by any EDC treatment nor by hCG injection (Tab. 12), however, variability was high. The only significant difference could be found in plasma T levels of hCG-injected and sham-injected frogs: sham-injected frogs exhibited much lower plasma T concentrations ($p \leq 0.01$; Fig. 29). For this analysis, corresponding controls were pooled ($n = 20$), because data did not differ significantly between the different exposure experiments.

Tab. 12: Plasma testosterone (T), estradiol (E2) and progesterone (P4) concentrations of *Xenopus laevis* (n = 10) exposed to methyldihydrotestosterone (MDHT), vinclozolin (VIN), ethinylestradiol (EE2), and tamoxifen (TAM). Frogs exposed to VIN and EE2 received an injection with 100 IU human chorionic gonadotropin (hCG; dissolved in 100 µL H_2O) before exposure. Corresponding controls were pooled (n = 20), because data did not differ significantly between the different exposure experiments. Given are means ± S.E.M. Treatments did not differ significantly from controls.

Treatment	**Exposure concentration** (M)	**Plasma T** (ng/mL)	**Plasma E2** (pg/mL)	**Plasma P4** (pg/mL)
Solvent control - hCG	–	19.0 ± 2.6	170.0 ± 12.0	175.2 ± 38.1
Solvent control + hCG	–	98.6 ± 26.8	193.7 ± 37.8	234.4 ± 41.3
MDHT - hCG	10^{-10}	26.7 ± 3.2	176.5 ± 12.3	133.6 ± 15.4
	10^{-8}	22.5 ±2.4	172.9 ± 14.9	142.3 ± 34.5
	10^{-7}	25.2 ± 3.5	175.9 ± 16.7	149.5 ± 37.5
VIN + hCG	10^{-10}	76.6 ± 12.3	169.5 ± 10.5	217.4 ±18.5
	10^{-8}	73.2 ± 15.6	167.8 ± 4.5	256.1 ± 33.0
	10^{-6}	73.5 ± 19.3	166.0 ± 8.4	248.9 ± 50.6
EE2 + hCG	10^{-10}	61.0 ± 25.6	175.0 ± 28.0	215.9 ± 32.2
	10^{-8}	81.5 ± 19.2	175.0 ± 13.3	209.0 ± 33.8
	10^{-6}	66.3 ± 7.2	236.4 ± 30.1	250.5 ± 23.8
TAM - hCG	10^{-10}	26.9 ± 4.3	195.8 ± 16.7	159.7 ± 17.1
	10^{-8}	48.4 ± 3.8	170.5 ± 22.3	151.2 ± 26.4
	10^{-7}	56.4 ± 7.6	158.2 ± 22.2	170.7 ± 19.4

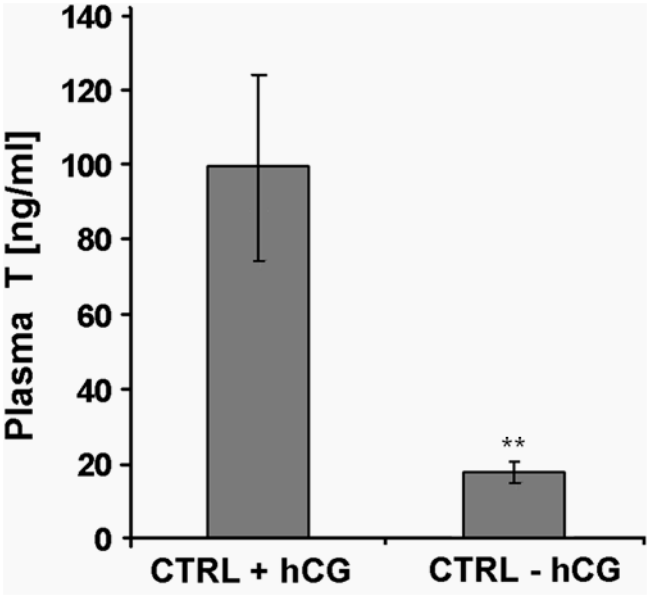

Fig. 29: Plasma concentrations of testosterone in *Xenopus laevis.* Because plasma T levels of samples of control frogs injected with 100 IU human chorionic gonadotropin (CTRL + hCG) and samples of frogs injected with water (CTRL – hCG), respectively, did not differ between the experiments, data was pooled (n = 20). Given are means ± S.E.M (** $p \leq 0.01$).

3.2.2 Gene expression analyses

Because individual variability was high, neither aromatase nor steroid reductase type 1 or steroid reductase type 2 gene expression of gonad samples was significantly influenced by any EDC treatment (Tab. 13). Likewise, LH and FSH gene expression of brain samples did not differ compared to controls (Tab. 13). Nevertheless, EE2 exposure at concentrations of 296 µg/L and 2.96 µg/L significantly induced Vtg mRNA expression ($P < 0.01$; Fig. 30), but no lower EE2 concentration (29.6 ng/L) affected Vtg mRNA expression (Fig. 30).

Tab. 13: Relative mRNA expression of aromatase (ARO) and steroid reductase type 1 (RED1) and type 2 (RED2) in gonad samples, as well as luteinizing hormone (LH) and follicle stimulating hormone (FSH) in brain samples of *Xenopus laevis* (n = 10) exposed to 17α-methyldihydrotestosterone (MDHT), vinclozolin (VIN), ethinylestradiol (EE2), and tamoxifen (TAM). Frogs exposed to vinclozolin and ethinylestradiol received an injection with 100 IU human chorionic gonadotropin (hCG; dissolved in 100 µL H_2O). Given are means ± S.E.M. Treatments did not differ significantly from controls.

	Treatment	Exposure concentration (M)	Gonad samples			Brain samples	
			ARO	RED1	RED2	LH	FSH
+ hCG	Solvent control - hCG	–	81.9 ± 19.7	93.6 ± 21.8	119.0 ± 28.3	77.8 ± 10.6	49.9 ± 13.3
	Solvent control + hCG	–	100.0 ± 14.6	100.0 ± 7.3	100.0 ± 10.7	100.0 ± 27.5	100.0 ± 38.7
	VIN	10^{-10}	88.2 ± 9.8	96.0 ± 8.0	99.4 ± 9.2	94.8 ± 26.7	75.5 ± 27.3
		10^{-8}	112.1 ± 13.6	108.5 ± 12.1	92.1 ± 17.0	114.6 ± 23.6	98.3 ± 73.3
		10^{-6}	128.3 ± 14.2	109.1 ± 8.6	110.2 ± 14.9	98.1 ± 22.6	101.0 ± 74.9
	EE2	10^{-10}	86.8 ± 8.9	111.3 ± 13.9	137.8 ± 12.3	101.4 ± 13.7	77.8 ± 10.7
		10^{-8}	85.8 ± 6.1	113.1 ± 7.9	139.3 ± 15.7	101.5 ± 20.8	59.1 ± 14.3
		10^{-6}	107.9 ± 10.7	98.4 ± 8.4	123.3 ± 11.0	89.2 ± 19.4	76.3 ± 19.6
- hCG	Solvent control - hCG	–	100.0 ± 17.5	100.0 ± 13.6	100.0 ± 24.4	100.0 ± 34.8	100.0 ± 26.2
	MDHT	10^{-10}	90.3 ± 13.1	103.8 ± 14.2	100.3 ± 26.6	105.0 ± 21.2	90.8 ± 27.1
		10^{-8}	95.6 ± 6.5	133.4 ± 16.3	106.9 ± 21.3	109.2 ± 29.4	88.3 ± 16.5
		10^{-7}	113.8 ± 16.4	126.3 ± 15.0	95.6 ± 18.1	124.9 ± 48.9	94.0 ± 23.9
	TAM	10^{-10}	130.0 ± 11.7	114.0 ± 9.1	116.6 ± 17.0	119.3 ± 28.4	104.7 ± 30.6
		10^{-8}	123.7 ±15.1	125.2 ± 11.0	108.4 ± 24.5	117.0 ± 37.4	89.7 ± 26.5
		10^{-7}	114.7 ± 9.8	106.5 ± 12.5	81.7 ± 20.2	127.8 ± 41.4	93.9 ± 30.2

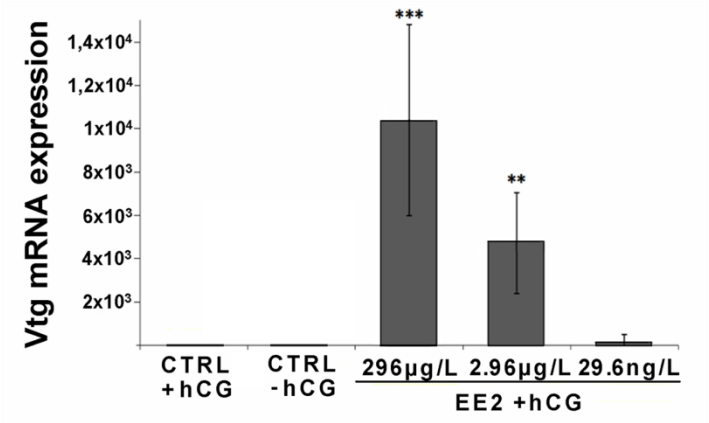

Fig. 30: Vitellogenin (Vtg) mRNA expression. Relative mRNA expression of hepatic Vtg in *Xenopus laevis* after a 96h exposure to 17α-ethinylestradiol (EE2) at three different concentrations (mean ± S.E.M.; n= 10 per treatment). Statistical differences were determined using one-way ANOVA followed by Dunnett T3 post-hoc tests. Normality of data was ensured using the Kolmogorov-Smirnoff test. Significant differences from control (CTRL) + human chorionic gonadotropin (hCG) are marked by asterisks (** $p \leq 0.01$; *** $p \leq 0.001$).

3.3 Reversibility of altered spectral and temporal parameters of male advertisement calls due to exposure to 17α-ethinylestradiol

Remarkably, the reduced number of accentuated clicks and the decreased duration of clicks within male ACs due to EE2 exposure persisted during four weeks under control conditions without any EE2 ($P < 0.05$; Fig. 31).

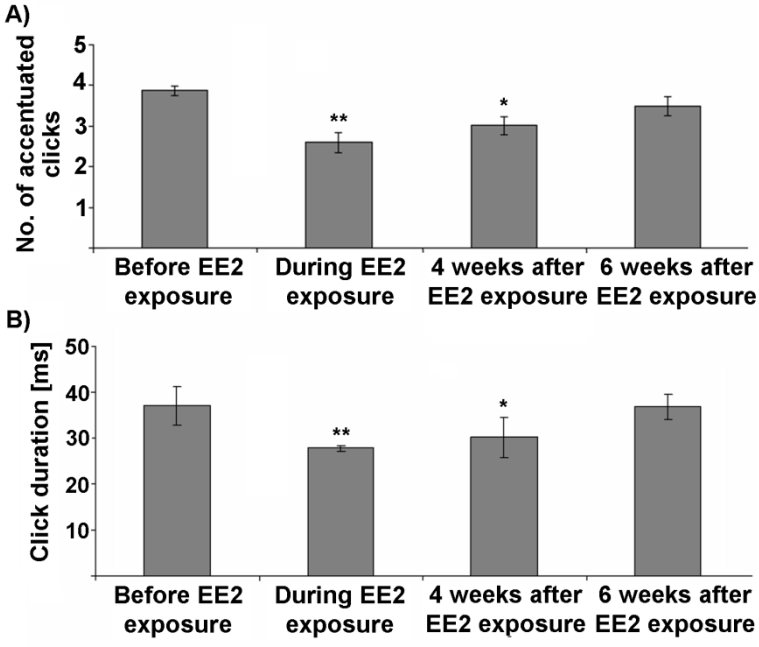

Fig. 31: Reversibility of call modifications due to 17α-ethinylestradiol (EE2) exposure. Median (IQR) (n= 10) of (A) no. of accentuated clicks and (B) duration of clicks within the advertisement calls before, during, and four and six weeks after EE2 exposure (at 2.96 µg/L). Statistical differences were determined using two-tailed Wilcoxon signed-rank tests for paired samples. To control for type I errors from conducting multiple tests, false discovery rate (FDR) was applied. Significant differences are marked by asterisks (* $p \leq 0.05$; ** $p \leq 0.01$).

After six weeks without EE2 exposure, however, the features did not show any differences to control levels any more (Fig. 31).

3.4 Biological relevance of alterations of spectral and temporal parameters of male advertisement calls due to 17α-ethinylestradiol exposure

When presented with recordings of male AC, female frogs were attracted to these vocalisations, as they spent more time near the speaker playing recorded male AC than with the speaker playing only noise (P = 0.003; Fig. 32 a). When having the choice between playbacks of AC of unexposed control males and playbacks of AC uttered by the same males while being exposed to EE2, female frogs were significantly more active when being close to the speaker playing AC of unexposed control males (P = 0.027; Fig. 32 b). Likewise, if presented with only one alternative playback at a time, females were also more active at the speakers when playbacks of control animals were played (P = 0.011; Fig. 32 c). All subjects ovulated within 12 h after hCG injection, indicating a sexually aroused state

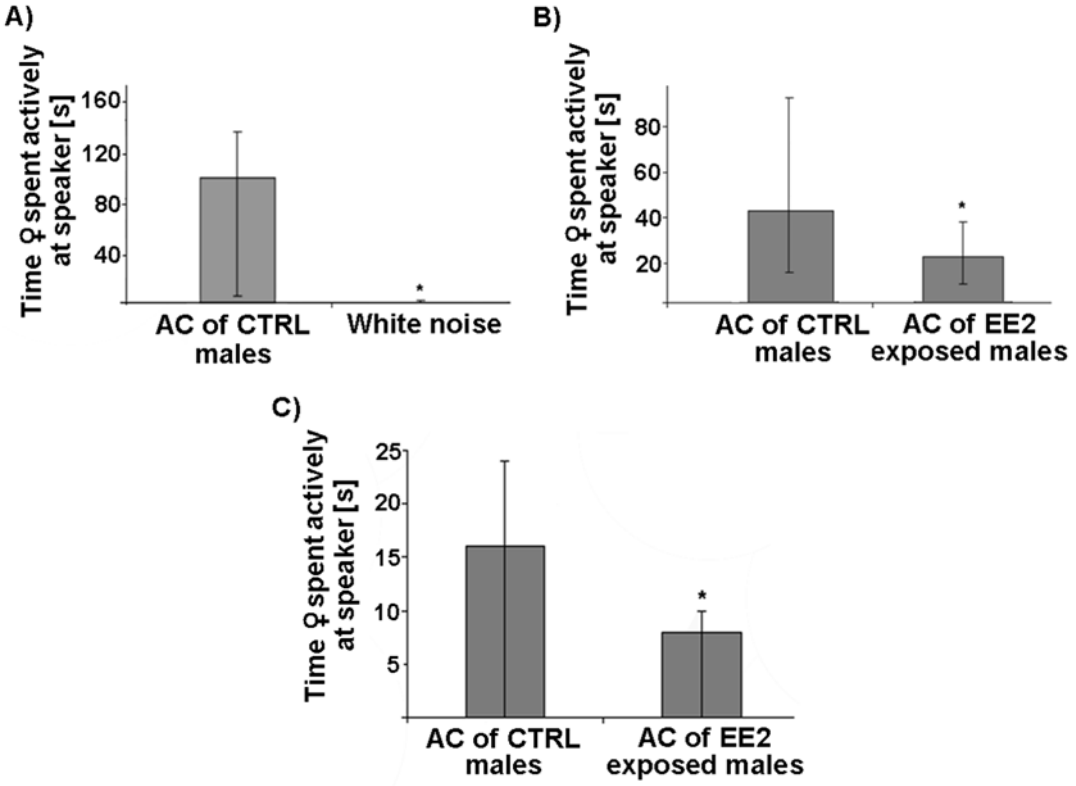

Fig. 32: Female choice experiments. (A) Time females spent actively at the speaker playing recordings of advertisement calls of unexposed control males versus white noise (median (IQR); n=10). (B) Time females spent actively at the speaker playing recordings of advertisement calls of unexposed control males and AC of the same males being exposed to 17α-ethinylestradiol (EE2) (2.96 µg/L), respectively (median (IQR); n=10). (C) Time females spent actively in the two arms when presented with only one playback at a time: a playback of advertisement calls of an unexposed control male or a playback of advertisement calls of the same male being exposed to EE2 (2.96 µg/L) (median (IQR); n=10). Statistical differences were determined using a two-tailed Wilcoxon-signed rank test for paired samples. Significant differences are marked by asterisks (* p ≤ 0.05).

4 Discussion

The general aim of this study was to introduce a non-invasive test method based on the calling behavior of male *X. laevis* which can be used as biomarker for the assessment of (anti)androgenic and (anti)estrogenic EDCs, distinguishing between these four different MOAs. Additional tests concerning the reversibility of potential effects of certain EDCs on male mate calling behavior, as well as the assessment whether modifications of male mating calls affect the attractiveness of males for females were performed to reveal the biological relevance of exposure of *X. laevis* to particular, environmentally relevant EDCs.

4.1 Androgenic 17α-methyldihydrotestosterone

Previously, it was shown that advertisement calling of male *X. laevis* is suppressed by castration and can be partly restored by exogenous androgen treatment (Wetzel and Kelley 1983). Accordingly, male frogs recorded in this study produced a higher percentage of advertisement calls when exposed to environmentally relevant concentrations of the xeno-androgen, MDHT, suggesting an increase in male sexual arousal. This effect might be due to direct action of MDHT on the male larynx, since these muscle fibers as well as the laryngeal motor neurons were shown to possess numerous AR (Kelley et al. 1975; Kelley 1981; Sassoon and Kelley 1986; Segil et al. 1987; Perez et al. 1996) which can be a direct target of androgen action (Kelley 1980; Gorlick and Kelley 1987; Segil et al. 1987; Kelley et al. 1989; Kelley and Tobias 1999). However, MDHT might also interfere with the vocal pathway of *X. laevis*, a defined neural circuit in the central nervous system, consisting of the VST, the APOA, the DTAM, the rRpd and neurons in the cranial nerve nucleus IX-X (Wetzel et al. 1985; Emerson and Boyd 1999; Brahic and Kelley 2003). The vocal pathway is responsible for generating mate calling behavior in *X. laevis* and neurons throughout this pathway express ARs (Kelley et al. 1975; Kelley 1981; Perez et al. 1996). Thus, neurons within this pathway could also be direct targets of MDHT action.

Although lower doses (1 mg hormone pellet implants) of the nonaromatizable androgen DHT did not significantly increase the advertisement calling of castrated male *X. laevis* in a previous study (Wetzel and Kelley 1983), this study showed that even environmentally relevant concentrations of a non-aromatizable xeno-androgen (up to 30.45 ng/L) can affect the calling behavior of male *X. laevis*. The increase in

the proportion of male advertisement calling due to MDHT exposure suggests that unexposed females, which are attracted to and sexually stimulated by male ACs during their fairly short periods of sexual receptivity (Picker 1980, 1983; Tobias et al. 1998b), might prefer MDHT exposed males, which broadcast enhanced sexual arousal, as it was shown for females of various amphibian species (Halliday and Tejedo 1995; Lopez 1996; Bourne 1998). However, if a pond is contaminated by MDHT it is not only males who are exposed to this EDC but also females are affected. Since a previous study demonstrated that MDHT exposure interrupts oogenesis (Cevasco et al. 2008) and affects reproductive biology (Massari et al. 2010) in female *X. laevis*, it is possible that MDHT exposure might reduce reproductive success of this species and thereby adversely affects population dynamics, although MDHT can increase male sexual arousal. However, it remains to be shown that female *X. laevis* exposed to MDHT alter their phonotactic and receptive behavior and further experiments are needed to evaluate whether (short-term) exposed pairs of frogs in fact have lower reproductive success than controls. Moreover, further studies are required to identify mechanisms of androgenic MDHT action at the larynx as well as in the vocal-motor pathway of the central nervous system.

4.2 Antiandrogenic vinclozolin

The obtained results demonstrate that VIN at all applied concentrations led to significant alterations of acoustic behavior of *X. laevis*, which are very much likely to result in reduced reproductive success. The diminished calling activity due to a decrease in utterance of ACs and chirping, which also indicates a reduced sexual arousal and the alteration of temporal and spectral features of the male advertisement call of VIN-treated frogs, might be the result of different inhibitory mechanisms of action of VIN. *X. laevis* is a fully aquatic organism and vocalizes under water without breathing. Clicks are produced by contractions of laryngeal muscles in response to the activity of the laryngeal nerves (Tobias and Kelley 1987; Kelley and Tobias 1999). Thus, the larynx of *X. laevis* is the effector organ for the male-specific vocalizations. Laryngeal muscle fibres of male *X. laevis* express high levels of AR (Kelley et al. 1975; Kelley 1981; Sassoon and Kelley 1986; Segil et al. 1987) and have been shown to be a direct target of androgen action (Kelley 1980; Gorlick and Kelley 1987; Segil et al. 1987; Kelley et al. 1989; Kelley and Tobias

1999). VIN, however, interferes with the negative feedback mechanism of T on pituitary gonadotropin secretion, and thereby affects T levels (O'Connor et al. 2002; Kubota et al. 2003; Loutchanwoot et al. 2008) and also inhibits androgen binding to AR (Wong et al. 1995; Kelce et al. 1997; Kelce and Wilson 1997). Therefore, it is most likely that VIN interferes with the direct action of androgen on the larynx and thereby reduces mate calling activity of male *X. laevis.* By the same mechanisms of action, VIN might also interfere with ARs that are expressed throughout the vocal pathway of *X. laevis*, and hence alter male mate calling behavior. Within the vocal pathway, especially the VST, which expresses high levels of gonadotropin receptors (Morell et al. 1975; Yang et al. 2007), was found to be a strong candidate for acoustic pattern modulation of vocalizations of male and female *X. laevis* (Yang and Kelley 2008). Androgen is predominantly produced by male gonads in response to gonadotropin released by the pituitary, which in turn is stimulated by GnRH from the hypothalamus (Yang and Kelley 2008). Hence, in addition to its effects on androgen secretion, gonadotropin itself may also have direct influence on vocalization by directly working on the brain (Yang et al. 2007). VIN has the potential to affect gonadotropin production via feedback on hypothalamus and pituitary, thus it might alter male mate calling parameters of *X. laevis* by indirectly altering VST action patterns via gonadotropins, as well as by directly acting on AR of the vocal pathway and the larynx.

In this study, adult *X. laevis* showed elevated testosterone levels after hCG injection, resulting in an elevated calling activity, which is in accordance with previous studies by Wetzel and Kelley (1983) demonstrating that vocalizations of sexually aroused male *X. laevis* require androgens. Because the antiandrogen VIN and its metabolites M1 and M2 are AR antagonists (Gray et al. 1994; Kelce and Wilson 1997; Wilson et al. 2007), a reduction in calling behavior following VIN exposure is expected, which has been shown by Behrends et al. (2010) for the antiandrogen FLU. As predicted, VIN also diminished vocalizations but only at the highest concentration (286.1 µg/L). FLU showed the same effect at a concentration of 2.76 µg/L (Behrends et al. 2010). This difference in effectiveness might be due to the lower efficiency of VIN (Kang et al. 2004). The decrease in calling activity due to VIN exposure could be ascribed to a diminished vocal utterance of calls that characterize a sexually aroused state of the males. Moreover, VIN at any of the applied concentrations led to increased relative proportions of call types, mostly growling, indicating a decrease in sexual arousal of

these males (Picker 1983; Tobias et al. 1998a and b; Vignal and Kelley 2007). Accordingly, the proportions of call types, which indicate a sexually aroused state of the male such as ACs and chirping, decreased at environmentally relevant concentrations. These observations are in agreement with an earlier study of Hannigan and Kelley (1986) suggesting that androgens could analogously suppress the ticking vocal pattern in clasping male *X. laevis* and facilitate mate calling behavior. The fairly short periods of female sexual receptivity (Kelley 1996) and the high population densities in the wild suggest that impairments of male mate calling behavior, especially a reduction of AC proportion, might result in a reduced reproductive success of VIN-exposed males.

This study also shows that VIN exposure at the concentrations of 286.1 µg/L, 2.86 µg/L and 28.6 ng/L decreases the mean click duration and the number of accentuated clicks of the slow trill parts of ACs but does not affect any other temporal and spectral parameters, such as ICD and fundamental frequency. In agreement with these results, Wetzel and Kelley (1983), who compared the fundamental frequency and the ICD of male *X. laevis* ACs before and after castration, did not find an impact of T treatment on any of the two parameters. However, in the same study the authors observed a significant increase of the mean ICD in hCG-treated frogs, which was not detected in this study. Nevertheless, previously it was shown that in several taxa, including anurans, temporal features of mate calling behavior carry information for temporal pattern identification and mate recognition (Littlejohn et al. 1960; Loftus-Hills and Littlejohn 1971; Gerhardt 1978; Picker 1983; Schul 1998; Fonseca and Revez 2002; Schul and Bush 2002; Sueur and Aubin 2003), whereas spectral parameters function as acoustical adornments (Vignal and Kelley 2007). Therefore, the reduction in click duration, as well as the decreased number of accentuated clicks induced by VIN exposure even at a concentration of 28.6 ng/L might result in reduced reproductive success. However, to confirm this, further studies and female choice tests are warranted.

4.3 Estrogenic 17α-ethinylestradiol

In male *X. laevis*, the relation between estrogen levels and courtship behavior is unclear. Estrogens were assumed to play no role in male calling activity (Wetzel and Kelley 1983), which was revised by these recent results. Prompt and significant impacts of EE2 exposure on the male mate calling behavior of *X. laevis* were found

already at environmentally relevant concentrations much below the threshold of the classical and highly sensitive estrogenic biomarker Vtg induction. The reduction of uttered proportions of AC and the increase of the relative amount of the call type rasping indicate a lowered sexual arousal of EE2 exposed males. This modification of behavior might be caused by altered relations between endogenous androgens and estrogens or disruptions of genomic or non-genomic signalling pathways (Watson et al. 2011) triggered solely by estrogens.

Remarkably, EE2 exposure concentrations as low as 0.296 ng/L also alter spectral and temporal parameters of AC of male *X. laevis* and these immediate effects remain over four weeks. They are completely reversed to control levels after six weeks, suggesting that EE2 impacts might be due to fast and long term alterations in the central vocal-motor pathway located in the central nervous system, as it was shown for estrogen-exposed females (Tobias et al. 1998a; Wu et al. 2001).
In birds, estrogens are assumed to contribute to the neural masculinization of the avian song system during a critical period of development (Konishi and Akutagawa 1988; Wade and Arnold 1996) and treatment of adult male starlings (*Sturnus vulgaris*) with estrogenic EDCs was shown to result in louder, longer and more complex birdsongs compared to control males (Markman et al. 2008). This effect was suggested to be due to an increase in volume of the principle nucleus in the songbird brain, the high vocal center (HVC) (Markman et al. 2008). Accordingly, EE2 might similarly affect size and number of neurons within nuclei of the vocal pathway of male *X. laevis* (Brahic and Kelley 2003) and thereby affect parameters of the male mate calling behavior, although the masculine pattern of muscle fibers necessary for song production in *X. laevis* are predominantly dependent on androgens (Marin et al. 1990).
Markman and colleagues (2008) further demonstrated that female starlings prefer the more complex songs of males which were exposed to higher concentrations of estrogenic EDCs, although exposed males showed reduced immune function. In contrast this findings demonstrate that female *X. laevis* are less attracted to AC of EE2 exposed males and perform courtship-specific behavior (positive phonotaxis) to a lesser extend when located in a pond with EE2 exposed males. Because sexual identity recognition of male and female frogs, as well as mate identification was shown to be based on temporal calling parameters (Loftus-Hill and Littlejohn 1971; Gerhardt 1978; Vignal and Kelley 2007), alterations of temporal or spectral

parameters due to EE2 contamination might disable females to discriminate properly within and between *Xenopus* species. Moreover, spectral cues of calls of male *X. laevis* convey information about the attractiveness of a potential mate (Vignal and Kelley 2007), thus modifications of spectral cues due to EE2 exposure might also be less attractive for selecting females.

According to the results, it was previously shown that courtship-specific behavior of male fish decreased after EE2 exposure at environmentally relevant concentrations (Colman et al. 2009; Saaristo et al. 2009, 2010a; Partridge et al. 2011) nearly as sensitive as for *X. laevis*. Saaristo and colleagues (2009, 2010a and b), for instance, demonstrated that Sand goby (*Pomatoschistus minutus*) males exposed to EE2 show impaired courtship behavior (11 ng/L) and fail in nest and mate competition (4 ng/L). Likewise, male guppy (*Poecilia reticulata*) exposed to EE2 at 100 ng/L built smaller nests and showed a decreasing trend in fertilization success (Montgomery et al. 2012). EE2 exposure (≥ 1 ng/L) even resulted in feminization of secondary sexual traits in male pipefish (*Syngnathus scovelli*), with males still being capable of reproduction, but females discriminating against exposed males (Partridge et al. 2011). These cumulative findings indicate that behaviors of aquatic vertebrates associated with courtship and mating can be adversely affected by extremely low concentrations of EE2, suggesting the possibility of a lower reproductive success of exposed animals. However, further studies are needed to clarify the impact of estrogenic EDC exposure on immune functions of male *X.* laevis. Moreover, additional experiments are necessary to elucidate whether additional parameters of the male mate calling such as amplitude, bout lengths or temporal sequencing of different call types are also affected by estrogenic and other EDCs.

This study demonstrates for the first time that EE2 affects drastically male mate calling behavior of *X. laevis*, although estrogens were previously assumed to play no at all in role in male calling (Wetzel and Kelley 1983). These reproducible effects occurred promptly (24 – 96 h) at extremely low and environmentally relevant concentrations so that no ‘lowest observed effect concentration’ (LOEC) or ‘no observed effect concentration’ (NOEC) could be determined, yet. The effects were moreover reversible after six weeks. Hence, the endpoint male calling behavior of *X. laevis* can indeed be used as an extremely sensitive, fast and reproducible biomarker for the assessment of estrogenic EDCs.

In humans, EE2 at higher dosages (0.1 mg / kg twice a day) has also been shown to reduce male sexual desire and activity (Bancroft et al. 1974; Murray et al. 1975). In many European countries, male human reproductive health has decreased during the last few decades (Toppari 2002). The declining semen quality, as well as the increasing occurrence of testicular cancer, hypospadias and cryptorchidism were suggested to be, at least in part, attributable to exposure to estrogenic EDCs during fetal and childhood development (Toppari et al. 1996; Toppari 2002). However, further data collections are needed to investigate, whether exposure to environmentally relevant concentrations of EE2 at different stages of development adversely affects sexual behavior of humans and mammals.

4.4 Antiestrogenic tamoxifen and fulvestrant

Sole TAM exposure neither affected any measured parameter of male mate calling behavior of *X. laevis*, nor any classical invasive biomarkers. In a previous study on *X. laevis*, TAM exposure was shown to affect Vtg and aromatase mRNA expression (Urbatzka et al. 2007, Massari et al. 2010), as well as plasma E2 levels and mRNA expression of LH and FSH (Urbatzka et al. 2006b, 2007) of female but not male *X. laevis.* Urbatzka and colleagues (2007) suggested that this discrepancy between the sexes might be due to the naturally low endogenous estrogen levels in males, which might be too low to be altered by TAM severely enough to affect estrogen feedback (Urbatzka et al. 2006b). The lacking effect of TAM within this study might also be due to this phenomenon.

Simultaneous exposure to EE2 and TAM resulted in fewer EE2 effects, however, the increased proportions of rasping, as well as the elevated numbers of accentuated clicks within ACs could not be obliterated. ICI, on the other hand, cancelled out any EE2 effects. TAM exhibits different mechanisms of action (Shou et al. 2004), e.g. it can be estrogenic, as well as antiestrogenic in various tissues (Webb et al. 1995; MacGregor and Jordan 1998; Bentrem et al. 2001; Shou et al. 2004,). This ability might be based on interactions between TAM and various proteins involved in the transcription of estrogen-responsive genes (MacGregor and Jordan 1998). The E2 analogue ICI, on the other hand, is a pure estrogen antagonist without any estrogenic properties (MacGregor and Jordan 1998) and greater ER affinity than TAM (Wakeling and Bowler 1987). ICI was previously shown to inhibit estrogen signaling through ER completely (Osborne et al. 1995; Wakeling 1995; Wardley 2002). Hence, the low

affinity of TAM to ER and/or its partial estrogen-like activity might be the reason for its lower potency to obliterate EE2 effects compared to ICI co-exposure.
Experimental results obtained by exposure to individual EDCs demonstrate that co-exposure to EDCs with different MOAs can have distinct, divergent outcomes (Kawahara and Yamashita 2000; Sun et al. 2009). Estrogenic effects, for example, can be neutralized or reinforced by antiestrogenic exposure, as it was shown in this and several other studies (Kawahara and Yamashita 2000; Sun et al. 2009). However, antiestrogenic exposure itself might not exhibit any effects. Thus, studies performing co-exposure to EDCs with different MOAs might help to understand combined effects of EDCs, as they are expected in real, natural exposure situations.

4.5 General discussion

Similarly to VIN exposed frogs, male *X. laevis* exposed to the estrogen EE2 were also less sexually aroused than control frogs. Androgen (MDHT) treated animals, on the other hand, displayed enhanced sexual arousal indicated by higher proportions of AC. Surprisingly, the anti-androgen VIN interfered with different call types in comparison to the androgen MDHT: while both, VIN and MDHT, affected advertisement calling, VIN affected in addition the call type growling, whereas MDHT interfered with the call type rasping (Fig. 33). The estrogen EE2, however, was shown to have impact on rasping as well (Fig. 33). Hence, rasping seems to be affected by the amount of free circulating androgens and estrogens and/or the relation between androgen and estrogen levels, while growling seems to be affected if androgen binding to the AR is competitively inhibited.

EE2 and VIN were also shown to alter temporal and spectral parameters of the male advertisement call of *X. laevis*, whereas MDHT did not affect any of these parameters (Fig. 33). This suggests that the presence of estrogens (EE2) as well as a decrease in ligand-free AR, as it is the case for VIN exposure, might interfere with the DTAM, VST, the Ri, the nerve nucleus IX-X and/or the rRpd, which are responsible for generating vocal activity patterns in the central vocal-motor pathway of male *X. laevis* (Morell et al. 1975; Yang et al. 2007, Yang and Kelley 2008). In the presence of androgens (MDHT) and ligand-free AR, however, these effects seem to be absent. Nevertheless, to identify the exact mechanisms of (anti)androgenic and estrogenic EDC action, further physiological and neurophysiological studies are needed.

Exposure to EDCs was shown to result in serious alterations of behavior of various vertebrates (Crisp et al. 1998; Crews et al. 2000; Clotfelter et al. 2004; Zala and Penn 2004; Kloas et al. 2009) and this study extends that knowledge by providing evidence for significant short-term behavioral effects of (anti)androgenic and (anti)estrogenic EDCs on reproductive behavior of male and female *X. laevis* (Fig. 33). The results of this study demonstrate that the behavior of aquatic vertebrates associated with courtship and mating is adversely affected by extremely low concentrations of the estrogenic EE2 and the antiandrogenic VIN, suggesting a lower reproductive success of exposed animals, which might contribute to the global problem of amphibian decline. MDHT treated frogs, on the other hand, displayed increased advertisement calling, indicating enhanced sexual arousal. However, MDHT exposed female *X. laevis* showed interrupted oogenesis (Cevasco et al. 2008) and impaired reproductive biology (Massari et al. 2010). Thus, it is possible that MDHT exposure might reduce reproductive success of exposed animals, although MDHT can enhance male sexual arousal. Treatment with the antiestrogen TAM did not affect any of the measured parameters of male mate calling behavior. However, if frogs are simultaneously exposed to the estrogen EE2 and TAM or the pure estrogen antagonist ICI, EE2 effects are cancelled out. Whether simultaneous exposure of male *X. laevis* to EDCs with different MOAs always leads to an obliteration of some EDC effects, or whether all exposure substances can act synergistically and affect male mate calling behavior in a completely different way, needs to be further investigated by examining various MOA in parallel reflecting wildlife situations. Moreover, previous studies demonstrated that androgenic (Andreoletti et al. 1983; Wetzel and Kelley 1983), as well as antiandrogenic (Behrends et al. 2010) and estrogenic (Xu et al. 2008; Saaristo et al. 2009, 2010a) treatments affect reproductive behaviors of aquatic vertebrates, but this is the first study, providing evidence that antiestrogenic EDCs can repress estrogen-induced behavioral effects in aquatic vertebrates.

The results obtained in this study affirm that the male mate calling behavior of *X. laevis* can be used as extremely sensitive biomarker for the detection of (anti)androgenic and (anti)estrogenic EDCs. Moreover, the parameters measured were shown to be able to distinguish between the different MOAs tested and to identify and characterize specific MOAs (cf. Fig. 33). The specific characteristics for androgenic (MDHT) exposure of male frogs, for instance, were the elevated proportions of ACs and the lower percentages of the call type rasping (Fig. 33). The

specific features of the antiandrogenic VIN, in contrast, were the higher percentages of the call type growling (Fig. 33). The elevated proportions of the call type rasping were the unique particularity of estrogen (EE2) exposed animals (Fig. 33), and antiestrogens (ICI) specifically extinguished estrogenic effects (Fig. 33).

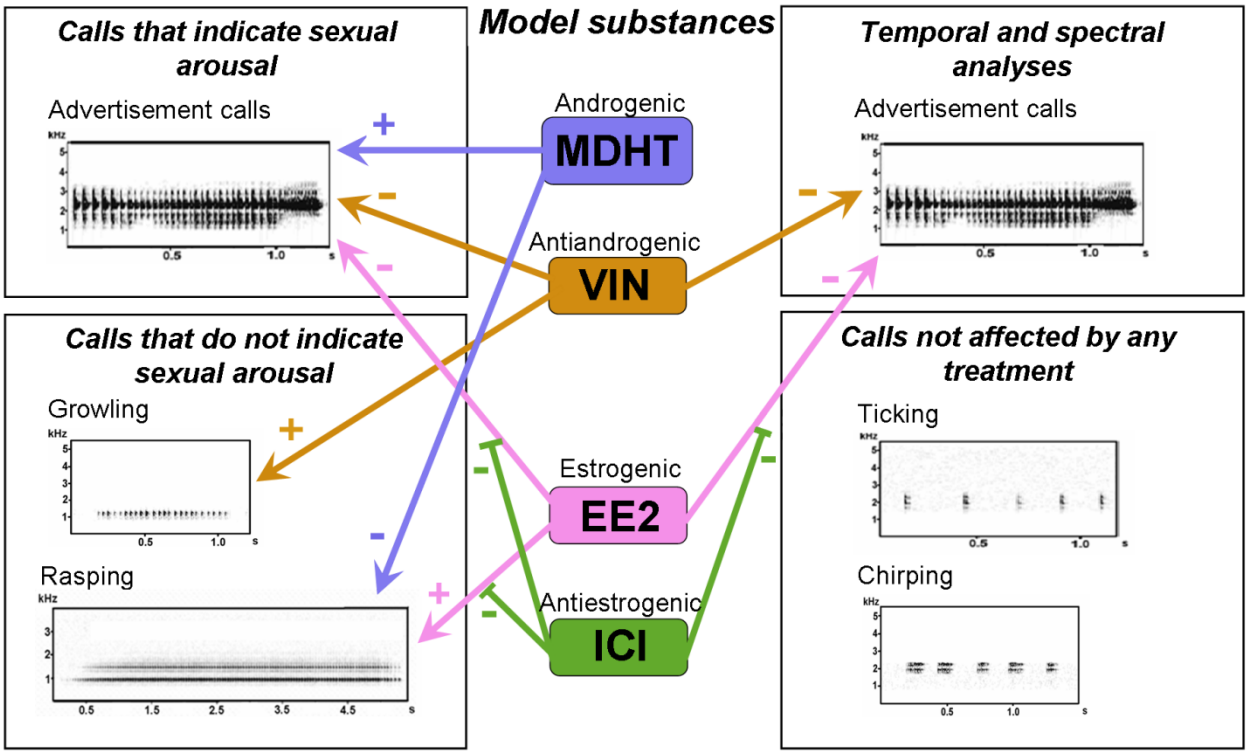

Fig. 33: Schematic diagram of the various effects of (anti)androgenic and (anti)estrogenic endocrine disrupting compounds. While the androgenic methyldihydrotestosterone (MDHT, blue color) increases the percentages of advertisement calls uttered and decreases the percentages of the call type rasping, the antiandrogen vinclozolin (VIN, orange color) decreases the relative proportions of advertisement calls and elevates the percentages of the call type growling. VIN furthermore decreases the number of accentuated clicks, as well as the click duration of clicks within advertisement calls (temporal and spectral analyses). The estrogenic 17α-ethinylestradiol (EE2, pink color) also lowers the relative amount of advertisement calls and reduces the number of accentuated clicks and the click duration. EE2, however, does not affect growling but lowers the call type rasping. When EE2 and the antiestrogenic fulvestrant (ICI, green color) are co-administered, ICI inhibits each EE2 effect.

Compared to the short-term behavioral endpoints used in this study, only one of the applied classical invasive biomarkers showed differences between EDC exposed and non-exposed frogs. Vtg mRNA expression was induced by EE2 exposure only at higher concentrations (296.4 µg/L and 2.96 µg/L), however, environmentally relevant concentrations of EE2 failed to induce Vtg mRNA expression after such a short exposure duration. Moreover, no further tested EDC (VIN, MDHT, TAM/ICI) affected any of the classical, invasive biomarkers tested.

Taken together, this newly established, behavioral and thus non-invasive method can be used as highly sensitive biomarker for the detection of EDCs, being the first biomarker that can differentiate between different MOAs when detecting EDCs of (anti)androgenic or (anti)estrogenic MOAs (Fig. 33). Current biomarkers cannot distinguish between, for instance, antiandrogenic and estrogenic MOAs: when determining sex ratio, estrogens and antiandrogens are changing sexual differentiation in the same direction towards feminisation (Kloas et al. 1999; 2002). Similarly, previous behavioral experiments determined suppressed or impaired reproductive behavior in fish exposed to antiandrogens and estrogens (Martinović et al. 2007; Sebire et al. 2008; Xu et al. 2008; Saaristo et al. 2009, 2010a; Partridge et al. 2011). However, none of these studies evaluated whether specific differences between the two MOAs could be detected.

Besides being able to differentiate between different MOAs, the herewith introduced new methodology is fast (1 - 4 days) and comparatively economical. Hence, the male mate calling behavior of *X. laevis* has huge potential to become a highly sensitive, standardized, non-invasive biomarker for the detection of (anti)androgenic and (anti)estrogenic EDCs.

5 Conclusions and Perspectives

The assessment of EDCs in aquatic life relies on biomarkers. However, to date, most of the existing biomarkers for the assessment of (anti)androgenic and (anti)estrogenic EDCs are invasive, molecular biological or biochemical techniques, resulting in irreversible impacts, or, like in most cases, in sacrifice of experimental animals during the analyzing processes (Kloas et al. 2009). Although reproductive behavior previously turned out to be a useful endpoint for the detection of some - especially estrogenic - EDCs (Bell 2001, 2004; Bjerselius et al. 2001; Zala and Penn 2004; Brian et al. 2006; Sebire et al. 2008; Colman et al. 2009; Behrends et al. 2010; Saaristo et al. 2010a and b; Partridge et al. 2011), until recently the use of behavior as endpoints for the assessment of EDCs has been neglected by ecotoxicologists (Clotfelter et al., 2004; Zala and Penn, 2004). No standardized behavioral test assessing (anti)estrogenic and (anti)androgenic EDCs exists yet. However, this study indicates that by using male mate calling behavior of *X. laevis* as endpoint, it is possible to evaluate environmentally relevant concentrations of EDCs, differentiating between different modes of action when detecting EDCs of (anti)androgenic or (anti)estrogenic MOA (Fig. 33). So far, no other sensitive biomarker for detecting (anti)estrogenic and (anti)androgenic EDCs has been shown to respond so specifically at such low concentrations and after such short exposure time (Jolly et al. 2006; Katsiadaki et al. 2006; Urbatzka et al. 2006 a and b, 2007; Sebire et al. 2008; Kloas et al. 2009; Behrends et al. 2010). Thus, the high sensitivity, and the capability of the method established here to differentiate between different modes of action when detecting EDCs indicate the huge potential for this rapid behavior test to become a sensitive, standardized, non-invasive biomarker with even diagnostic value. However, to develop a standardized test guideline based on the male mate calling behavior of *X. laevis*, the applied method would have to be optimized: first, data analysis ought to be automatized. By programming software that automatically measures parameters of acoustic recordings, including total calling activity, proportions of the different call types used, duration of AC clicks, number of accentuated clicks within ACs, as well as further spectral and temporal parameters (e.g. frequency, bandwidth, entropy, etc.) data analysis would be less time consuming and entirely standardized. Second, since in this study EDC effects could be shown after only one day of exposure, the exposure duration might also be reducible to optimize this procedure. And third, one of the most important

contributions to standardization would be the application of a flow-through system. Semi-static exposure of EDCs, as it was performed in this study, usually results in decreased nominal concentrations in the test tanks after short periods of time (Levy et al. 2004). A flow-through system would eliminate this problem, because a certain nominal EDC concentration can be maintained and thereby sensitivity might be increased. However, the lowest EDC concentrations tested in this study still revealed significant results, thus another further step towards standardization of this method would be to determine NOECs and LOECS (lowest observed effect concentrations) for each particular EDC used.

In addition to standardizing this non-invasive method for the assessment of (anti)androgenic and (anti)estrogenic EDCs presented here, the same technique might be used to assess multiple stressors and further ecological aspects, such as light pollution or climate change.

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Annex

Annex 1 - EIA Kit Protocol (Cayman Chemical Manual)

The 96-well plate(s) included with this kit is supplied ready to use. Each plate must contain a minimum of one total activity well (TA), two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B_0), and an eight point standard curve run in duplicate (S1 – S8). Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate.

1. Add 100 mL EIA buffer to NSB wells. Add 500 µL EIA buffer to B_0 wells.
2. Add 50 µL from standard tube #8 to both of the lowest standard wells. Add 50 µL from standard tube #7 to each of the next two standard wells. Continue with this procedure until all standards are aliquoted.
3. Add 50 µL of sample per well.
4. Add 50 µL to each well *except* the TA and the Blk wells.
5. Add 50 µL to each well *except* the TA, the NSB, and the Blk wells.
6. Cover each plate with plastic film and incubate one hour at room temperature on an orbital shaker.
7. Reconstitute Ellman's Reagent immediately before use. Empty the wells and rinse five times with Wash Buffer. Add 200 µL of Ellman's Reagent to each well. Add 5 µL of tracer to the TA wells. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. These assays typically develop in 60 – 90 min.
8. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc. Remove the plate cover being careful to keep Ellman's Reagent from splashing on the cover. Read the plate at a wavelength between 405 nm and 420 nm. The absorbance may be checked periodically until the B_0 wells have reached a minimum of 0.3 A.U. (blank substracted). The plate should be read when the absorbance of the B_0 wells are in the range of 0.3 – 1.0 A.U. (blank substracted). If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman's Reagent and let it develop again.

Annex 2 - Isolation of total RNA from tissue samples (gonads, brains, livers)

1. Homogenize tissue in 700 µL Qiazol (Qiagen, Hilden, Germany) using a Tissue Lyser (2 x 1.5 min at 20.0 1/s).
2. Add 600 µL Qiazol to each sample and centrifuge at 12.000 g (10 min, 4 °C). Incubate samples at room temperature for 10 min.
3. Transfer 1000µl of the clear Qiazol fraction into a new 2 mL tube and add 300 µL fresh Qiazol.
4. Add 250 µL Chloroform and vortex tubes for exactly 15 s. Incubate samples at room temperature for 10 min.
5. Centrifuge at 12.000 g for 15 min (4 °C). Transfer 400 µL of the clear aqueous phase into a new 2 mL tube.
6. Add 400 µL ice-cold Isopropanol and mix gently. Incubate samples for 10 min at room temperature.
7. Centrifuge samples at 12.000 g for 15 min (4 °C). Discard supernatant and remove all Isopropanol.
8. Wash pellet by adding 300 µL ice-cold EtOH. Mix gently.
9. Centrifuge samples at 12.000 g for 15 min (4 °C). Discard supernatant and remove all EtOH.
10. Dry pellets at room temperature for 10-15 min.
11. Dissolve pellets in 12 – 17 µL DEPC-H_2O (according to pellet size).
12. Freeze samples at -80 °C until further processing.

Annex 3 - Agilent 2100 Bioanalyzer, RNA 6000 Nano LabChip Kit Protocol

1. Take out RNA Nano 6000 reagents (stored at 4 °C) and place them in the dark at room temperature for 30 min before starting.
2. Dispense enough RNA 6000 ladder (Ambion, stored at -70 °C) for use in one day (1 µL per chip) into a tube. Follow the same procedure for each RNA sample to be loaded (1 µL per lane, at 5 – 500 ng/µL). Since your sample may evaporate when heated, a 2 µL aliquot is a good idea.
3. Decontaminate the electrodes on the bioanalyzer before and after each chip run. Each box of chips contains two Electrode Cleaner chips; label these for use with either RNaseZap or RNAse-free DI water. They can be used for cleanings for the entire box of chips.
    a. Slowly pipet 350 µL RNAseZap and 350 µL RNAse-free DI water into any one well of the appropriate Electrode Cleaner chip. The liquid should spread to cover the bottom of all wells.
    b. Place the RNAseZap Electrode Cleaner chip into the bioanalyzer, close the lid, and incubate for 1 min. Open the lid and remove the chip.
    c. Place the RNAse-free DI water Electrode Cleaner chip into the bioanalyzer, close lid, incubate for 10 sec. Open lid and remove the chip.
    d. Allow the bioanalyzer to dry with open lid for at least 10 sec before use.
    e. The filled Electrode Cleaner chips can be used multiple times during the day but should be emptied at the end of the day.
4. Prepare Gel Matrix by filtering 550 µl in the supplied column. Centrifuge at 1500 g (4000 rpm in Eppendorf microcentrifuge) for 10 min. Dispense 65 µl filtered gel into Eppendorf tubes and store at 4 °C. Use within one month.
5. Vortex the Dye Concentrate for 10 sec and spin down. Add 1 µL to a 65 µL gel matrix aliquot. Cap, vortex thoroughly, and spin at 13,000g for 10 min. Return dye concentrate to 4 °C in the dark.
    a. Shield the gel mix from light whenever applicable.
    b. Try not to introduce bubbles.
    c. Use gel-dye mixture within the same day.
6. Take a new chip out of its bag and place it on the chip priming station. Pipette 9 µL of gel-dye mix into the well marked G (in a black dot).
    a. Draw gel-dye mix from top of reagent to avoid any debris after spinning.

b. Keep the pipette tip flush to bottom of well when dispensing to avoid introducing bubbles.
c. Replace syringe on the chip priming station with each new LabChip kit.

7. Set timer to 1 min and pull the plunger up to the 1 mL mark. Close the chip priming station so that the latch audibly clicks. Depress the plunger, tucking it under the clip. Wait exactly 30 sec and release. If the syringe gasket is properly sealed, the plunger should quickly (in < 1 sec) jump past 0.3 mL mark and then slow down. Wait for 5 sec, then slowly pull plunger up to the 1 mL mark. Open the chip priming station.
8. Pipette 9 µL gel-dye mix into the remaining wells marked G (in gray dot). Discard the remaining geldye mix.
9. Pipette 5 µL Nano Marker (buffer) into all 12 sample wells and the ladder well.
10. Denature the ladder and RNA samples for 2 min at 70 °C before loading them. Quick-spin them down to collect the evaporation.
11. Pipette 1 µL ladder into the well marked with a ladder.
12. Pipette 1 µL sample into each sample well. If a well is empty, add 1 µL DI water or marker to maintain volume; otherwise the chip will not read correctly.
13. Place the chip on the vortexing station by first tucking one edge against the bump towards the front. Vortex the chip for 1 min at 240 rpm. There should not be any liquid coming out of the chip; wipe off any visible liquid before placing the chip in the bioanalyzer. Begin the run within 5 min to avoid evaporation and loss of volume in wells.
14. Activate the software before inserting the chip. On the bottom right corner, the software will recognize the bioanalyzer and show an icon. If it doesn't, make sure the bioanalyzer is on and plugged into the proper port (COM1).
15. Open the lid and insert the chip. It fits only one way, force is unnecessary. Close the lid SLOWLY.
16. The software will recognize the chip and show the appropriate icon at the upper left. If it doesn't, you will have to prepare another chip.
17. Select: *Assays* → *Electrophoresis* → *RNA* → *Eukaryote Total RNA* (for example). Then click *Start* to begin the run.

Annex 4 - DNase treatment protocol

1. Dilute samples using RNase free-DEPC water, until RNA concentration is 1 µg RNA / 8 µl.
2. Incubate 2 µg RNA (16 µL of diluted sample) DNase I working solution, consisting of 1 µL RNase free water (Qiagen, Hilden, Germany), 2 µL reaction buffer (10x; Invitrogen, Karlsruhe, Germany) and 1 µL DNase I (1 unit / µL) at room temperature for 15 min.
3. Add 2 µL EDTA (100 mM; Invitrogen, Karlsruhe, Germany) to inactivate DNase I and incubating the solution for 10 min at 60 °C.

Previous studies, as well as preliminary experiments demonstrated that DNase I treatment does not affect RNA quality.

Annex 5 – Effects of exposure to different concentrations of tamoxifen (TAM) on male calling behavior of *Xenopus laevis.*

Tab. A1: Effects of exposure to different concentrations of tamoxifen (TAM) on male calling behavior of *Xenopus laevis.* Values are median (IQR). Treatments did not differ significantly from controls.

Treatment	Night (after exposure)	Advertisement call (%)	Growling (%)	Ticking (%)	Rasping (%)
Solvent control - hCG injection	1	83.1 (75.3 – 86.7)	0.1 (0.4 – 1.6)	0.1 (0.1 – 0.2)	15.4 (11.8 –
	2	79.1 (73.0 – 86.9)	0.4 (0.3 – 1.0)	0 (0 – 0.1)	20.9 (12.2 –
	3	87.5 (77.3 – 90.7)	0.3 (0.2 – 1.5)	0 (0 – 0.1)	9.8 (9.1 – 21.0)
	4	82.2 (68.8 – 86.7)	0.7 (0.3 – 0.9)	0 (0 – 0.1)	15.6 (12.4 –
TAM 37.1 µg/L	1	94.9 (82.1 – 96.3)	0.6 (0 – 1.5)	0 (0 – 0)	3.4 (2.3 – 13.2)
	2	98.1 (96.4 – 100)	0.3 (0 – 0.4)	0 (0 – 0)	0.1 (0 – 2.3)
	3	91.9 (87.3 – 95.3)	0.5 (0 – 0.8)	0.1 (0 – 1.7)	5.4 (1.8 – 7.0)
	4	92.7 (89.6 – 95.1)	0.6 (0 – 1.0	0 (0 – 0.1)	6.8 (4.3 – 9.8)
TAM 3.71 µg/L	1	86.1 (84.0 – 93.8)	0.3 (0 – 0.7)	0 (0 – 0.1)	13.4 (5.7 –
	2	86.7 (84.0 – 88.6)	1.0 (0.3 – 2.1)	0 (0 – 0)	11.8 (9.3 –
	3	88.6 (82.0 – 90.0)	0.4 (0.2 – 1.8)	0 (0 – 0)	11.2 (8.1 –
	4	87.3 (85.1 – 88.3)	0.8 (0.4 – 6.8)	0 (0 – 0)	10.0 (3.4 –
TAM 37.1 ng/L	1	89.4 (88.2 – 92.1)	0.4 (0.2 – 0.7)	0 (0 – 0.1)	9.9 (7.0 – 11.1)
	2	89.8 (87.7 – 92.3)	0.4 (0.1 – 1.0)	0 (0 – 0.01)	9.2 (6.8 – 11.8)
	3	89.8 (88.9 – 92.8)	0.8 (0.6 – 1.8)	0 (0 – 0.2)	8.8 (6.3 – 9.3)
	4	90.2 (86.8 – 92.8)	1.0 (0.5 – 1.9)	0.1 (0 – 0.2)	8.0 (6.2 – 12.2)

Publications

Peer-reviewed Journals

1. Hoffmann F, Musolf K, Penn D (2009) Freezing urine reduces its efficacy for eliciting ultrasonic vocalizations from male mice. Physiol Behav 96 (4-5), 602 – 605.
2. Kloas W, Urbatzka R, Opitz R, Würtz S, Behrends T, Hermelink B, Hoffmann F, Jagnytsch O, Kroupova H, Lorenz C, Neumann N, Pietsch C, Trubiroha A, VanBallegooy C, Wiedemann C, Lutz I (2009) Endocrine Disruption in Aquatic Vertebrates. Trends Comp Endocrinol Neurobiol 1163, 187 – 200.
3. Musolf K[1], Hoffmann F[1], Penn D (2010) Ultrasonic courtship vocalizations in wild house mice, *Mus musculus musculus.* Anim Behav 79(3), 757 – 764.
([1]These authors made equal contributions)
4. Hoffmann F, Kloas W (2010) An environmentally relevant endocrine-disrupting antiandrogen, vinclozolin, affects calling behavior of male Xenopus laevis. Horm Behav 58(4), 653 – 659.
5. Hoffmann F, Musolf K, Penn DJ (2011) Spectrographic analyses reveal signals of individuality and kinship in the ultrasonic courtship vocalizations of wild house mice. Physiol Behav 105(3), 166 – 771.
6. Hoffmann F, Musolf K, Penn DJ (2011) Ultrasonic courtship vocalizations in wild house mice: Spectrographic analyses. J Ethol 30, 173 – 180.
7. Hoffmann F, Kloas W (2012) Effects of environmentally relevant concentrations of the xeno-androgen, methyldihydrotestosterone, on male and female mating behavior in *Xenopus laevis.* Chemosphere 87(11), 1246 – 1253.
8. Hoffmann F, Kloas W (2012) Estrogens can disrupt amphibian mating behavior. PLoS One 7(2), e32097. Doi: 10.1371/journal.pone.0032097.
9. Hoffmann F, Kloas W (2012) The synthetic progestogen, levonorgestrel, but not natural progesterone, affects male mate calling behavior of *Xenopus laevis.* Gen Comp Endocrinol 176(3): 385 – 390.

Oral and poster presentations

1. Hoffmann F, Musolf K, Penn D (2006) It's all about freshness. XXI International BioAcoustic Congress, Pavia, Italy (poster presentation).

2. Hoffmann F, Kloas W (2011) Calling behavior of male *Xenopus laevis* is affected by a xenoestrogen. 7th International Symposium on Amphibian and Reptilian Endocrinology and Neurobiology (ISAREN), Ann Arbor, Michigan, USA (oral presentation).

3. Hoffmann F, Kloas W (2011) Calling behavior of male *Xenopus laevis* is affected by a xenoestrogen. The inaugural meeting of the North American Society for Comparative Endocrinology (NASCE 2011), Ann Arbor, Michigan, USA (oral presentation).

4. Hoffmann F, Kloas W (2011) Xenoestrogens disrupt calling behavior of South African clawed frogs *(Xenopus laevis)*. 8th International Conference on Behaviour, Physiology and Genetics of Wildlife, Berlin, Germany (poster presentation).

5. Hoffmann F, Kloas W (2001) Amphibian behavior - a new biomarker to assess endocrine disruptors affecting reproduction. Environment and Public Health in Modern Society - EPH*modern* 2011 - Second EPH Annual Meeting, Potsdam, Germany (poster presentation).

Further publications and media echo

1. Hoffmann F, Kloas W (2010) Wenn Frösche nicht flirten. Jahresforschungsbericht IGB-Berlin.

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Des Weiteren erkläre ich meine Kenntnisnahme der dem angestrebten Verfahren zugrunde liegenden Promotionsordnung. Ich habe mich anderweitig nicht um einen Doktorgrad beworben und bin nicht im Besitz eines entsprechenden Doktorgrads.

Berlin,

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